# Redefining the role of tumour necrosis factor in macrophage differentiation and effector function in bacterial and tumour defences

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University of Tasmania Animal Ethics Committee permit numbers:
A13933, A13934, A13936

## Publications

**Part of the work contained in this thesis has been published or submitted for publication as detailed below:**

Chapter 3 is based on the published paper
**Li, X.,** Lyons, A. B., Woods, G. M., & Körner, H. (2017). The absence of TNF permits myeloid Arginase 1 expression in experimental L. monocytogenes infection. *Immunobiology*, *222*(8), 913-917.
XL performed the laboratory analysis, prepared tables and figures, drafted and refined the manuscript.

Chapter 5 is based on the ‘to be submitted' paper
**Li, X.,** Körner. H, Darby. J, Lyons, A.B. Woods, G.M. TNF signalling downregulates phagocytosis of tumour cells by activated macrophages, to be submitted.
XL performed the laboratory analysis, prepared tables and figures, drafted and refined the manuscript.

## Conference presentations

**Part of the work contained in this thesis has been presented at national and international conferences as detailed below:**

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**Abstract**

Tumour necrosis factor α (TNF) is an essential pro-inflammatory cytokine predominantly secreted by macrophages that regulates the inflammatory response to infection. Macrophages are essential phagocytic innate immune cells resident in all tissues. In response to distinct stimuli, macrophages are polarized into classically activated macrophages (CAM) or alternatively activated macrophages (AAM). CAM have a pro-inflammatory phenotype with a strong ability in killing invading pathogens whereas AAM display an anti-inflammatory phenotype and promote tissue repair. The enzymes iNOS and Arginase-1 are signature molecules expressed by CAM and AAM, respectively, and they use the same substrate L-arginine competitively. TNF plays a central role in restricting AAM differentiation in parasitic infection with *Leishmania (L.) major* and a tumour model and we hypothesized that it would be applicable generally. Therefore, this thesis investigated the role of TNF in the activation of macrophages during an infection with the gram-positive bacterium *Listeria (L.) monocytogenes*.

Wild-type and B6.TNF$^{-/-}$ mice were infected with *L. monocytogenes* and macrophage differentiation in spleen (Chapter 3) and peritoneum (Chapter 4) was analysed. TNF deficiency resulted in high susceptibility of infected mice to *L. monocytogenes* with high bacterial loads in spleen and liver. In addition, there was a strong expression of Arginase-1 and an intact presence of iNOS in splenic and peritoneal macrophages, indicating a regulation of AAM differentiation during *L. monocytogenes* infection by TNF. Furthermore, in response to the alternative activation of macrophages in B6.TNF$^{-/-}$ mice, the number of splenic neutrophils and the titre of the pro-inflammatory cytokine IL-1β increased. This indicates that TNF also orchestrates immune responses to bacterial infection and indicates that its role in the suppression of central molecules of the AAM signature such as Arg1 is a general phenomenon.

In addition, this thesis investigated the role of TNF in phagocytosis of *L. monocytogenes* and Devil Facial Tumour Disease (DFTD) cells by macrophages (Chapter 5). We examined macrophage phagocytosis of *L. monocytogenes* and DFTD cells by macrophages from wild-type and B6.TNF$^{-/-}$ mice. Under steady state conditions there was no difference in phagocytosis efficiency. However, the treatment of macrophages with IFNγ plus LPS or LPS alone increased phagocytosis in macrophages from B6.TNF$^{-/-}$ mice, to a greater degree than macrophages from wild type mice. It is likely that TNF regulates phagocytosis of DFTD cells by activated macrophages.

In summary, these results indicate a key role for TNF in orchestrating innate immune responses through modulating the macrophage differentiation and phagocytosis. The AAM bias in the absence of TNF leads to the high susceptibility to bacterial infection, suggesting an essential effect of TNF in an anti-bacterial response.

# Chapter 1

# Literature Review

## Chapter 1: Literature review

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## 1.1 Tumour necrosis factor

### 1.1.1 TNF in pathology

Tumour necrosis factor α (TNF) is an important inflammatory cytokine, which was first named in 1975 for its ability to induce tumour cell hemorrhagic necrosis in mice [5]. TNF has also been reported to play a role in a central endogenous mediator of endotoxic shock [5]. Additionally, as a pro-inflammatory cytokine, TNF is essential in initiation and resolution of inflammation. Overproduction of TNF is involved in many inflammatory diseases. Studies in TNF- or TNFR- deficient mice have indicated the essential role of TNF in host defence against bacterial and viral infections [5]. TNF is synthesised rapidly after infection and orchestrates pro-inflammatory cytokine production, which suggests its importance in the development of many inflammatory diseases including Rheumatoid arthritis (RA) and Crohn's disease [6]. For instance, high concentrations of TNF have been found in the joints and synovial fluid from RA patients [7]. Thus, drugs targeting TNF have been developed for treatment of RA and Crohn's disease. These include anti-TNF antibody (Remicade), and anti-TNF receptors Fc fusion protein (Etanercept, Lenercept) [8].

### 1.1.2 TNF expression

TNF is one of the 19 TNF family members which also includes TNFβ, FAS ligand (FasL) and CD40 ligand (CD40L) [5]. TNF genes are located within the cluster of major histocompatibility complex (MHC) genes on the murine chromosome 17 and the short arm of human chromosome 6 [9]. There is a 20-30% identity in protein sequence between family members and they share the conserved region referred to as the TNF homology domain (THD) [10]. THD is required for TNF family members to bind to the TNF receptors at conserved cysteine rich regions to activate downstream pathways. TNF is sourced from macrophages, mast cells, T cells and B cells [11]. Lipopolysaccharide (LPS) or TNF itself is the potent stimuli to TNF synthesis [11]. Several transcription factors regulate TNF expression including nuclear factor kappa B (NF-κB), activating protein-1(AP-1) and AP-2 [12]. There are two forms of TNF in cells: transmembrane (mTNF) and soluble TNF (sTNF) [13]. TNF is initially produced as a 27kD mTNF anchored by a N-terminal hydrophobic domain [14]. TNF is subsequently cleaved into 17kD sTNF, by the metalloprotease TNFα-converting enzyme (TACE) [15]. The expression of TNF is also regulated at the post-transcriptional level, with mRNA stability regulated by Tristetraprolin (TTP) [16].

### 1.1.3 TNF signal transduction

TNF acts through binding with the receptors, TNF receptor 1 (TNFR1) and TNF receptor 2 TNFR2, in a trimeric manner [6]. TNFR1 and TNFR2 are expressed in most mammalian cells [17]. TNF binds to TNFR1 with higher affinity than TNFR2 [18], suggesting the predominant role of TNFR1 in TNF induced cellular responses. The trigger of TNF signaling pathway leads to various responses, including cell survival, apoptosis and necrosis.

#### 1.1.3.1 Activation of the NF-κB and MAPK pathways

Following TNF binding, TNFR1 provides a docking site and interacts with the TNF receptor associated death domain (TRADD) [5]. TRADD then recruits TNFR-associated factors (TRAFs), which include TRAF2, TRAF5, receptor interacting protein-1 (RIP-1) and cellular inhibitor of apoptosis proteins 1 and 2 (cIAP-1 and 2), to form the TNF receptor signalling complex [5]. TRAF2 and TRAF5 double knockout cells demonstrated that TRAF2 and TRAF5 are required in TNF-induced NF-κB activation [19]. RIP-1 is a unique serine/threonine kinase with a death domain in the C-terminus [20]. The recruitment of RIP1 and TRAF2 are independent during the process of forming the signalling complex [21]. In addition, the lipid rafts provide the micro domain for the ubiquitylation of TNFR1 and RIP-1, as inhibition of the organization of lipid rafts impaired the NF-κB activation [22]. After formation of the TNF receptor signalling complex, RIP1 is polyubiquitinated by cIAP-1 and cIAP-2. This triggers activation of TGF-β activated kinase (TAK1) [22]. TAK1 then activates NF-κB which leads to NF-κB translocation into the cell nucleus, where it activates the transcription of genes such as cytokines, chemokines, growth factors and survival genes [23]. TAK1 activation also results in the activation of MAPK pathway which includes JNK and p38 [24]. The activation of MAPK pathway results in the activation of AP-1, it has similar activity to NF-κB in regulation of gene expression [5].

#### 1.1.3.2 Induction of apoptosis and necrosis

TNF induces cells to undergo apoptosis. The Fas-associated protein-containing death domain (FADD) is also recruited by TRADD and subsequently RIP-1 is deubiquitinated. FADD and RIP-1 activate pro-caspase 8 and in turn results in the release of cytochrome c from chondriosome [25]. The released cytochrome c leads to apoptosis through activating caspase-3/7 [25]. The activation of NF-κB inhibits TNF induced apoptosis, suggesting there is a balance between apoptosis and cell survival signals in response to TNF [26]. In the initiation of necrosis,

RIP-1 interact with RIP-3 to form the RIP-1/RIP-3 necrosome. RIP-3 then phosphorylate phosphoglycerate mutase family member 5 (PGAM5), which dephosphorylates dynamin-related protein 1 (Drp1) and leads to necrosis [27]. Knockdown of PGAM5 or Drp1 impairs TNF induced necrosis [5]. In addition, the generation of reactive oxygen species (ROS) also plays a role in PGAM5/Drp1 dependent TNF activated necrosis [28].

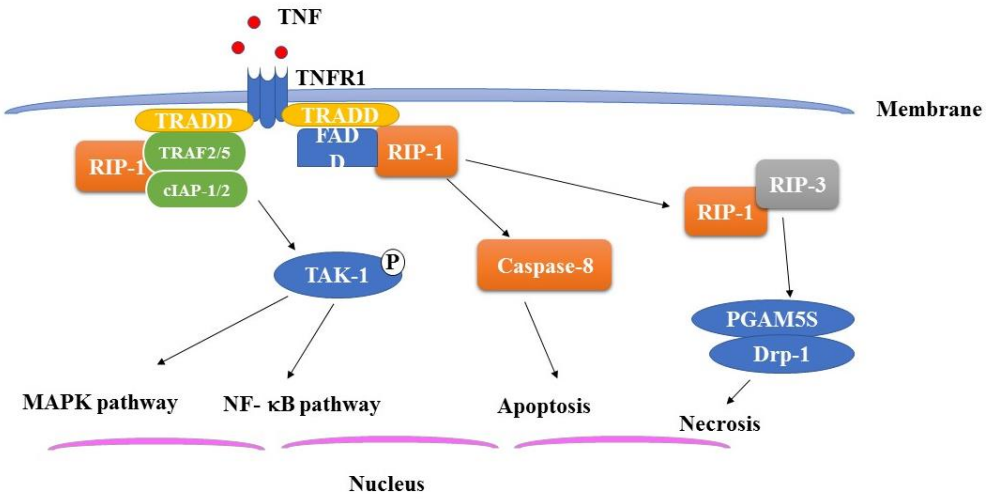


**Figure 1.1: Overview of TNF signaling pathway.** TNF binds with TNFR1 and then TNFR1 recruits TRADD. TRADD activates the formation of TNF receptor signalling complex, RIP-1 is subsequently triggers TAK-1 activation and leads to the activation of NF-κB and MAPK pathway. FADD interacts with RIP-1 and leads to the activation of caspase-8, then triggers the induction of apoptosis. RIP-1/RIP-3 necrosome formation leads to the phosphorylation of PGAM5S and subsequent necrosis. Adapted from [6].

### 1.1.4 TNF and macrophages

Macrophages are the dominant source of TNF during infection and injury [6]. TNF activates macrophages in an autocrine manner as a positive feedback loop to induce further activation of macrophages [29]. Macrophages are a population of innate immune cells that form the first line of defense against bacterial infection [6]. They are also essential in tissue homeostasis, tissue repair, regulation of adaptive immune response and inflammation [6]. Macrophages are a major defense system against invading microorganisms through phagocytosis, destruction of organisms, antigen presentation, and synthesis of a range of products, including enzymes, complement components, chemokines and cytokines [6]. The released cytokines and chemokines also activate macrophages in an autocrine/paracrine manner to further regulate the inflammatory response [6]. Because of the essential role of macrophages in physiological and

pathophysiological processes, mechanisms of TNF activities in macrophage have been investigated.

TNFR1 deficient mice are susceptible to the infection by pathogens, including *Leishmania major* [30], *Listeria. monocytogenes* [31]. It indicates the requirement of TNF signaling in host defense against parasite and bacterium. TNF is known in the activation of macrophages. In combination with IFNγ, TNF activates macrophages of classically activated macrophage phenotypes with a high capacity for microbicidal or tumoricidal, secreting pro-inflammatory cytokines [6]. Typically, TLR ligands trigger macrophages in a MyD88-dependent pathway to produce TNF, which cooperates with IFNγ to activate macrophages in an autocrine manner [32]. Some TLR ligands can also trigger the production of IFNβ by regulating the IFN-regulatory factor 3 (IRF3) pathway [33]. The IFNβ replaces IFNγ and combines with TNF to induce the classically activated macrophages [33]. In addition to the role of promoting differentiation of classically activated macrophages, TNF has been shown to inhibit alternatively activated macrophage differentiation [34, 35]. In infection by *L. major*, macrophages from $TNF^{-/-}$ mice were indicated to exhibit the phenotype of AAM, with high expression of AAM markers, such as Arginase-1, CD206, Ym1 and Fizz1 [35]. TNF inhibits AAM markers expression in IL-4 induced macrophages [35]. The role of TNF in inhibiting AAM differentiation in other model needs to be investigated.

### 1.2 Macrophages

Macrophages are resident in all tissues of adult mammals and they are heterogeneous in function in particular microenvironment. They have different names in different tissue, such as microglial cells in nervous system, Kupffer cells in liver, alveolar macrophages in lung, and Langerhans cells in skin [36]. Macrophages play key roles in maintance of tissue homeostasis by clearing apoptotic cells, tissue development, and immune response to pathogens by initiating and resolving inflammation [37]. Tissue resident macrophages regulate homeostasis by responding to changes to physiology and challenges from microenvironment. Macrophages can also be recruited from bone marrow or tissue resident progenitors by local proliferation in the situation of infection. Macrophages are important therapeutic targets in many diseases and related to many disease state. It makes the investigation of diverse phenotype in macrophages to be important.

### 1.2.1 Tissue resident macrophage origins

Tissue resident macrophages have at least three lineages in mice, which arise at different developmental stages and persist to adulthood [38]. Macrophages are generated from the yolk sac in the embryo and colonise in the tissue where they persist in the adult [39]. The fetal liver is responsible for a second linage and gives rise to circulating monocytes during embryogenesis [40]. After birth, bone marrow is the main source of circulating monocytes and tissue resident macrophages [40]. Bone marrow derived monocytes originate from hematopoietic stem cells (HSC) with self-renewing ability [41]. After a series of differentiations, HSC become granulocyte and monocyte progenitors (GMP) [42]. GMPs then differentiate into monocyte and dendritic cell progenitors (MDP) [42]. These generate DCs and macrophages and lose the ability to differentiate into granulocytes [43]. Yolk sac and fetal liver are the main contributers of tissue resident macrophages in homeostasis. It is evident that adult microglial cells are generated from yolk sac and Langerhans cells in skin are derived from the yolk sac and fetal liver [37]. These yolk sac derived macrophages express high levels of the F4/80 marker (F4/80$^{hi}$) in spleen, peritoneum, liver, skin and brain [44]. Under inflammatory conditions, bone marrow derived monocytes are recruited into inflammation sites and give rise to inflammatory dendritic cells (DC) and macrophages [41]. Due to the limitations of experimental approaches, the function of distinct source of macrophages in each tissue are unknown.

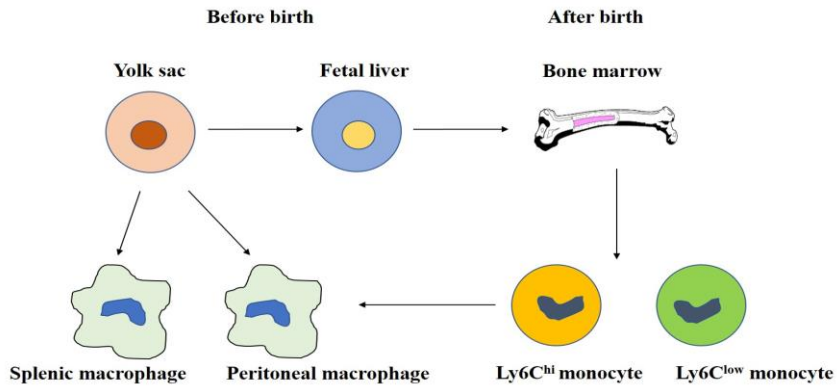


**Figure 1.2: Tissue resident macrophage origins in mice.** The tissue resident macrophages in adults originate from three sources. Before birth, yolk sac generates progenitors and populates the spleen and peritoneum. The second is fetal liver through progenitors generated from yolk sac. After birth, bone marrow is the main origin of tissue resident macrophages. The circulating Ly6C$^{hi}$ and Ly6C$^{low}$ monocytes give rise to macrophages and DCs in the tissue. Adapted from [1].

#### 1.2.1.1 Splenic macrophages

The spleen is a key organ for pathogen filtration, erythrocyte homeostasis and iron metabolism. It has all the major types of phagocytes such as monocytes, macrophages, and DCs [45]. These phagocytes are essential protectors in identifying cellular stress, pathogens, and phagocytosing dying cells [45]. The spleen is organized as red pulp, white pulp and marginal zones (MZ) [46]. Red pulp filters blood and recycles iron from old erythrocytes through phagocytosis by red pulp macrophages. White pulp has T cell and B cell follicles and protects the hosts against blood-borne pathogen infections and generates antigen specific immune responses [45]. The phenotype of red pulp macrophages is $F4/80^{+}CD206^{+}CD11b^{lo/-}$, and white pulp macrophages are identified by the pan-macrophage marker CD68 [47]. MZ surrounds the white pulp and promotes T cell responses by capturing antigens to white pulp [46]. MZ macrophages are characterized by the expression of MARCO (Macrophage receptor with collagenous structure), CD204 (Scavenger receptor A) and SINGR1 [47]. These MZ macrophages are efficient in trapping blood-borne antigens and pathogens. For example, SINGR1 recognizes polysaccharide antigens of *Mycobacterium tuberculosis* efficiently, then degrade these pathogens by phagocytosis [48].

#### 1.2.1.2 Resident macrophages in peritoneum and central nervous system

The peritoneal cavity has the cell composition of B cells, T cells, NK cells, macrophages, eosinophils and DCs [1]. There are two subsets in peritoneal macrophages, large peritoneal macrophages (LPMs) and small peritoneal macrophages (SPMs) [1]. LPMs are $F4/80^{high}CD11b^{+}$ and SPMs are characterized as $F4/80^{low}CD11b^{+}$ phenotype [49]. CD11b belongs to the integrin family and it forms the complement receptor 3 heterodimer together

**Table 1.1 | Phenotypic profile of LPMs and SPMs[1]**

| Surface molecule | LPMs | SPMs |
|---|---|---|
| F4/80 | +++ | + |
| CD11b | +++ | + |
| MHC-II | + | ++ |
| Ly6C | - | - |
| CD62L | - | ++ |
| CD80 | ++ | + |
| CD86 | +++ | + |

with CD18 [50]. CD11b is expressed not only on macrophages, but also DCs, T and B cells [50]. It is required for monocyte adhesion with other immune cells, as the deficiency of CD11b leads to impaired myeloid cell recruitment in inflammation [51]. F4/80 has been used as a specific mouse macrophage marker and it is a glycoprotein that belongs to epidermal growth factor-transmembrane7 (TM7) family [1]. It is expressed in tissue resident macrophages (Kupffer cells, microglia, Langerhans cells), eosinophils and DCs, but not on fibroblasts or lymphocytes [51].

LPMs are the more abundant peritoneal macrophages in homeostasis [1]. LPMs and SPMs are present in the peritoneal cavity from several mouse strains, such as C57Bl/6, BALB/c, 129/S6, SJL/J, FVB/N [1]. These two peritoneal macrophage subsets have distinct morphologies and phenotypes. LPMs have abundant vacuoles in the cytoplasm, whereas SPMs exhibit dendrites with a polarized morphology in culture [52]. In addition, LPMs and SPMs express different levels of surface molecules (Table 1.1). LPMs and SPMs exhibit different phagocytic abilities, cytokines synthesis and nitric oxide production [49]. Phagocytosis analysis showed that SPMs appear to have higher efficiency in phagocytosis of zymosan and *E. coli* [49, 52]. SPMs and LPMs don't produce significant levels of inflammatory cytokines in steady-state conditions. SPMs secrete increased levels of pro-inflammatory cytokines such as TNF, IL-1β and IL-12 following stimulation with *Staphylococcus epidermidis* cell-free (SES) supernatant [53]. During stimulation with LPS *in vitro*, SPMs produce TNF, MIP-1α whereas LPMs secrete G-CSF, GM-CSF [53]. SPMs from *Trypanoso macruzi*, zymosan-inoculated mice produce high levels of NO in response to LPS *in vitro* [52]. In the LPS inoculated mice, SPMs produce larger amounts of NO than LPMs [49].

After inflammatory stimulations, peritoneal cell subsets are altered dramatically, such as recruited monocytes, increased number of SPMs, and disappearing LPMs [54]. These responses are described as 'Macrophage disappearance reactions' [54]. The macrophage disappearance reactions is attributed to cell death, migration to lymph nodes or increased tissue adherence [1]. LPMs are generated from yolk sac and maintained by self-renewal. SPMs originate from bone marrow derived inflammatory monocytes in homeostasis [1]. Yolk sac generated macrophages in general are F4/80$^{hi}$ phenotype, which correlates with the LPM phenotype. During inflammatory conditions, SPMs differentiate from bone marrow derived Ly6C$^{+}$ monocytes [1]. Confirming that impaired inflammatory monocyte recruitment in CCR2$^{-/-}$ mice leads to reduced numbers of SPMs in inflammation [55]. After inoculation with LPS or thioglycollate, monocytes shift towards the SPM phenotype, suggesting a monocyte origin of

SPM [49]. LPMs undergo proliferation in homeostasis to maintain the number of F4/80$^{hi}$ peritoneal macrophages [1]. The proliferation ability of LPM is decreased in 12-week-old mice compared with newborn mice [56]. The transcription factor GATA-binding protein 6 (GATA6) is essential in LPM proliferation [55]. GATA6 is specifically expressed in LPMs and GATA-6 deficiency in macrophages leads to reduced number of LPMs in the peritoneal cavity [55]. Moreover, vitamin A induced retinoic acid induces the specific gene expression of GATA-6 in LPMs [55]. The self-proliferation of LPMs is a phenotype of AAMs which is related to tissue repair after resolution of the infection [57]. The macrophages in central nervous system, including microglia also have the ability to proliferate. Microglia are derived from yolk sac macrophages during early embryogenesis and express macrophage-associated markers, including CD11b and CD14 [58]. The self-proliferation of microglia is essential in infection, injury and irradiation throughout life. The transcription factor PU.1 and interferon regulatory factor 8 (IRF8) are implicated in microglia differentiation and development [59].

### 1.2.2 Molecules related to monocyte development

Monocyte development requires the stimulation of growth factors. Macrophage colony-stimulating factor-1 receptor (M-CSFR) is crucial for monocyte development. M-CSFR is a class III transmembrane tyrosine kinase receptor and it is expressed on most mononuclear phagocytic cells [41]. Macrophages are severely depleted in many tissues in M-CSFR deficient mice [60]. One of the M-CSFR ligands is M-CSF, the administration of M-CSF increases the proliferation of tissue resident macrophages, and expansion of the monocyte pool in tissues [61]. The other ligand of M-CSFR is IL-34, which is required for the development of microglia and Langerhans cells [62]. Granulocyte-macrophage colony-stimulating factor (GM-CSF) is the other growth factor that regulates macrophage development, as shown by GM-CSF deficient mice having defects in tissue macrophages (except for alveolar macrophages) [38]. In addition, the development of monocytes is regulated by several transcription factors. The transcription factor PU.1, belonging to Ets family, is required for monocyte generation [63]. PU.1 deficiency leads to a general myeloid lineage defect in mice [64]. PU.1 drives GMP development into monocytes through interaction with transcription factor C/EBPα [65], Gata-1 and Gata-2 [66]. The transcription factor Gata-6 is required for the local proliferation of resident macrophages [67]. Gata-6 deficiency leads to dysregulation of peritoneal macrophage proliferation in homeostasis and inflammation [67].

### 1.2.3 Monocyte heterogeneity in mouse

Monocytes are a population of innate immune cells which are highly heterogeneous in phenotype and function [41]. There are two subsets of monocytes in mice: Ly6C$^{hi}$ inflammatory and Ly6C$^{low}$ non-inflammatory monocytes [68]. Ly6C$^{hi}$ inflammatory monocytes express high level of CC- chemokine receptor 2 (CCR2) and low level of CX3C- chemokine receptor 1(CX3CR1) [68]. The Ly6C$^{low}$ non-inflammatory monocytes have low expression of CCR2 and high level of CX3CR1 [68]. The Ly6C$^{hi}$ monocytes and Ly6C$^{low}$ monocytes exhibit distinct functions with Ly6C$^{hi}$ monocytes being pro-inflammatory and Ly6C$^{low}$ monocytes having anti-inflammatory activities.

In bacterial (*L. monocytogenes* [69]) and protozoal (*Leishmania major* [70]) infections, Ly6C$^{hi}$ monocytes are recruited to the infected sites with a high capacity for producing TNF and inducible nitric oxide (iNOS). Conversely, Ly6C$^{low}$ monocytes patrol blood vessels to scavenge dead cells, oxidized lipids, and pathogens in homeostasis [71]. To carry out these roles, Ly6C$^{low}$ monocytes require integrin LFA-1 and chemokine receptor CX3CR1 [71]. During *L. monocytogenes* infection, Ly6C$^{low}$ monocytes are recruited in the very early stage of infection and mediate early responses to bacterial infection [71]. In the spinal cord injury model, Ly6C$^{low}$ monocytes are essential in wound recovery with high expression of IL-10 and arginase 1 [72]. Some studies have indicated that Ly6C$^{hi}$ monocytes are progenitors of Ly6C$^{low}$ monocytes with decreasing expression of Ly6C [73]. Ly6C$^{hi}$ monocytes differentiate into Ly6C$^{low}$ monocytes in homeostasis as demonstrated by a fate mapping study [40]. The M-CSFR antibody blockade results in an increased number of Ly6C$^{hi}$ monocytes and reduced number of Ly6C$^{low}$ monocytes [74]. The decrease of Ly6C$^{low}$ monocytes is balanced by the increase of Ly6C$^{hi}$ monocytes which suggested the requirement of M-CSF in the maturation of Ly6C$^{hi}$ to Ly6C$^{low}$ monocytes [74].

### 1.2.4 Monocyte migration in infection

Chemokines are small molecules required for the trafficking of monocytes into inflammatory sites. According to the sequence of cysteine residues in the ligands, chemokines are classified into four subsets: CC, CX3C, CXC and C; Where C represents a cysteine and X represents a noncysteine amino acid [75]. CCR2 is essential for the migration of Ly6C$^{hi}$ monocytes to the sites infected with *L. monocytogenes* [76], *M. tuberculosis* [77] and *L. major* [78]. The recruitment of Ly6C$^{hi}$ monocytes in response to *L. monocytogenes* infection was dampened in the CCR2 deficient mice [76]. CC-chemokines ligand 2 (CCL2) and CCL7 are ligands of

CCR2 and mediate the recruitment of Ly6C$^{hi}$ monocytes [79]. *L. monocytogenes* infection induces CCL2 and CCL7 production in the serum, liver, spleen and lung [79]. The enhanced CCL2, CCL7 expression in tissue results in the recruitment of inflammatory cells to infection sites [79]. The mechanism of CCL2 action in monocyte migration remains unclear. One idea is that the dimerization and glycosaminoglycan of CCL2 in specific tissues are required for gradient establishment and monocyte migration [75, 80]. The prevention of CCL2 dimerization or glycosaminoglycan leads to the impairment of monocyte recruitment. The recruitment of Ly6C$^{low}$ monocytes is mediated through CX3CR1 [81]. Deficiency of CX3CR1 leads to diminished Ly6C$^{low}$ monocyte recruitment [71]. The ligand of CX3CR1, CX3CL1 is upregulated in the marginal zone of the spleen in response to *L. monocytogenes* infection. It mediates early migration of Ly6C$^{low}$ monocytes into spleen [81]. Moreover, CX3CR1 has some roles in Ly6C$^{hi}$ monocyte migration as CX3CR1 deficiency also leads to the reduced migration of Ly6C$^{hi}$ monocytes to the spleen following bacterial infection [81].

### 1.2.5 Macrophage polarization and functional phenotypes

Macrophages adopt several functional phenotypes in response to the tissue microenvironment *in vivo*. They recognize pathogens to initiate and direct T-cell dependent immune responses [38]. The activated T cells release cytokines such as IFNγ and IL-4 to further activate macrophages [38]. In response to stimulation, macrophages can be polarized into classically activated macrophages (CAM, M1 macrophages) and alternatively activated macrophages (AAM, M2 macrophages) [2]. Macrophages are polarized toward CAM in the activation of IFNγ alone or in concert with TNF or LPS. Conversely, Th2 cytokines such as IL-4 and IL-13 polarize macrophages toward to AAM phenotype [2]. CAMs and AAMs exhibit different functional phenotypes in terms of receptor expression, effector function and cytokine production as discussed later.

#### 1.2.5.1 Classically activated macrophages

Upon activation, CAMs produce high levels of pro-inflammatory cytokines (TNF, IL-1β), chemokines, costimulatory molecules (CD80, CD86) and effector molecules (NO, ROS) [32]. IFNγ and LPS induce macrophages to express chemokines CXCL10, CXCL9 and CCL5 [2]. The expression of these chemokines is dependent on LPS through the activation of the transcriptional factor ‘signal transducer and activator of transcription 1’ (STAT1) [82]. In addition, the IFNγ and TLR signalling dependent expressions of CXCL10, CXCL9 and CCL5

are inhibited by IL-4 [82]. The suppression of transcriptional activation of IFNγ and LPS by IL-4 requires 'signal transducer and activator of transcription' (STAT6), which inhibits the activation of STAT1 and NF-κB [83]. NK cell production of IFNγ is required for the maturation of $Ly6C^{hi}$ monocytes following *L. monocytogenes* infection [84]. The NK cell secreted IFNγ is transient and the maintaining CAM activation needs adaptive immune responses [84]. TNF only activates macrophages in the presence of IFNγ, and the activated macrophages produce TNF and the new synthesised TNF activates macrophages with IFNγ in an autocrine manner [32]. IFNγ or TNF deficient mice are susceptible to *L. monocytogenes* infection, suggesting the requirement of both IFNγ and TNF in response to bacterial infection [85]. LPS combines with IFNγ and induces CAMs through toll like receptor 4 (TLR4) signalling [32]. Gene expression profiles of IFNγ in combination with LPS are different with the treatment of IFNγ, LPS alone [86]. IFNγ and LPS produce high levels of TNF in macrophages, as IFNγ enhances the transcription and mRNA stability of TNF [87]. These pro-inflammatory factors activate T cells and orchestrate a fully-activated inflammatory response.

Nitric oxide (NO) is an essential anti-microbial mediator produced by CAMs, which enables the capacity of macrophages to eliminate intracellular pathogens [69]. NO is a water soluble gas and it reacts with oxygen to exhibit microbial toxicity [88]. The enzyme of NO synthesis is NO synthase (NOS), which has three forms in mammals: NOS1, inducible NO synthase (iNOS or NOS2), and NOS3 [89]. NOS1 and NOS3 are constitutive forms and produce low amounts of NO in healthy hosts [90]. iNOS is the main enzyme involved in the production of NO under inflammatory conditions [90]. IFNγ, TNF, LPS induces the expression of iNOS in macrophages, often in a synergistic way [91]. *L. major* infected macrophages release TNF, which activates macrophage production of high levels of NO. This occurs in synergy with IFNγ via an autocrine mechanism [92]. The expression of iNOS in IFNγ/LPS activated macrophages requires TNF, as anti-TNF antibody blocks NO release upon IFNγ/LPS activation [93]. TNF induces the expression of iNOS through the activation of NF-κB [94, 95]. iNOS is beneficial in the defence against *L. monocytogenes* infection, as iNOS deficient mice have increased susceptibility to this pathogen [96]. However, some evidence suggests that NO increases *L. monocytogenes* survival by delaying phagolysosomes fusion in LPS activated macrophages [97]. The mechanisms by which NO in macrophage eliminates *L. monocytogenes* remains to be identified.

### 1.2.5.2 Alternatively activated macrophages

Th2 cytokines IL-4 and IL-13 induce macrophages into AAMs by binding the membrane receptor IL-4Rα and IL-13Rα1 respectively [98]. The transcription factor STAT6 is activated to regulate gene expression, including Arg1, CD206, Resistin-like molecule (Fizz1), and Chitinase 3-like 3 (Ym1) [99]. These genes are restricted to the mouse, since the human genome either lacks the homologs or they are dysfunctional [100]. Transglutaminase 2 (TGM2) exists in both the mouse and human genomes and it is recognized as the AAM marker in both species [20]. The high expression of mannose receptor CD206 in response to IL-4/IL-13 has been employed as a marker of AAMs. CD206 is widely expressed in tissue resident macrophages, lymphocytes and DCs [101]. IL-4/IL-13 induced AAMs have enhanced mannose-receptor-mediated phagocytosis [102], suggesting it is involved in macrophage phagocytosis of pathogen [101]. Fizz1 is highly induced by IL-13 and it is a secreted protein expressed by macrophages and eosinophils. Fizz1 deficiency in mice increases lung inflammation during *Schistosoma mansoni* infection [103]. Fizz1$^{-/-}$ AAMs promote the differentiation of antigen-specific Th2 cells [103]. The enzyme arginase-1(Arg1) is upregulated in AAMs and it is a competitor of iNOS in L-arginine metabolism. IL-4 induces the expression of Arg1 in the STAT-6 dependent manner [104]. TLR pathway is also reliant on the Arg1 production in the infection of intracellular pathogens such as *Toxoplasma gondii* [105]. iNOS converts L-arginine into nitric oxide and Arg1 converts L-arginine into urea and L-ornithine [69, 106]. The competition of iNOS and Arg1 in using limited L-arginine substrates shifts the metabolic pathway. The metabolism of L-arginine in macrophages is a key defining feature in CAMs and AAMs. NO is a key effector molecule in cell proliferation inhibition and microbicidal activity, whereas AAMs production of L-ornithine promotes tissue repair and cell proliferation [107]. Arg1 suppresses T cell proliferation by depleting arginine in the microenvironment [108], and promotes wound repair by converting arginase into collagens and increasing the availability of fibroblasts [109]. In addition to the phenotype markers, AAM have anti-inflammatory functions with high production of the cytokines IL-10 and TGF-β [110]. Macrophages produce IL-10 through the activation of TLR, C-type lectin and glucocorticoids [111]. IL-10 is essential in the regulation of several infections, as IL-10$^{-/-}$ mice have a lethal immunopathology following the infection of *Toxoplasma gondii* [112] and malaria [113]. IL-10 inhibits the production of chemokines and prevents the recruitment of DCs during mycobacterial infection, thus leading to the failure of Th1 cell differentiation [114]. IL-10 suppresses pro-inflammatory responses by inhibiting CD4$^{+}$ T cell proliferation and the

inflammatory cytokine expression (IFNγ, TNF) [115]. TGF-β induces collagen synthesis in fibroblasts and it is required in wound healing [116].

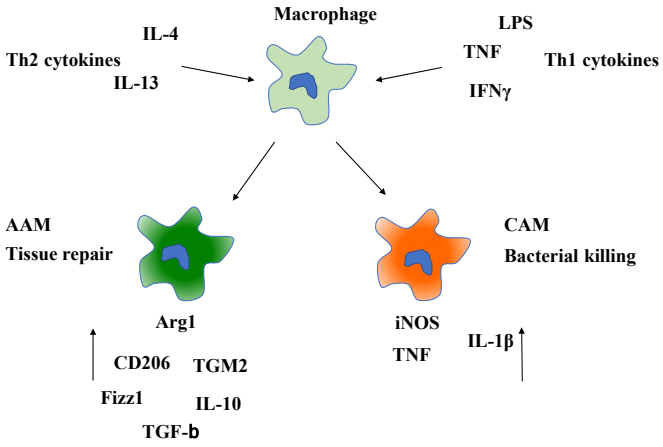


**Figure 1.3: Properties of polarized macrophages.** Classically activated macrophages (CAM) are activated by IFNγ and TNF, and LPS. Their phenotypes are characterized by the capacity for bacterial killing and production of pro-inflammatory mediators (TNF, IL-1β, iNOS). Conversly, alternatively activated macrophages (AAM) are induced by IL-4/IL-13. They express high levels of surface markers(Arg1, CD206, TGM2, Fizz1), and anti-inflamatory cytokines (IL-10, TGF-β). Adapted from [2].

### 1.2.5.3 Macrophage and cancer

Macrophages play a key role in tumour cell migration, invasion and angiogenesis. High density of tumour-associated macrophages (TAMs) occurs in several human cancers, such as breast, bladder and prostate cancer [2]. This suggests that macrophage infiltration is essential in tumour progression [117]. The inflammatory cytokines (IFNγ, TNF) produced by macrophages sustain the chronic inflammation in the tumour initiation and invasion [118]. After tumour establishment, TAMs change their phenotype to promote tumour progression [2]. The invasion and proliferation ability of tumour cells are enhanced when culturing with macrophages as macrophage secreted substances (MMP-9, TNF, TGF-β) stimulate tumour promotion [117]. They share the same characteristics with AAMs, with high expression of the surface markers (Arg1, Fizz1, Ym1) and release of the cytokines (IL-10, TGF-β) [119]. Generation of new blood vessels in and around the tumour is required for tumour progression [2]. TAMs promote angiogenesis by producing angiogenesis-modulating enzymes such as MMP-9, MMP-2 and cyclo-oxygenase-2 (COX-2) [120]. In addition, tumour cells express high levels of integrin

associated protein(CD47) to inhibit phagocytosis of TAMs [121]. Application of monoclonal antibody of CD47 enhances macrophage phagocytosis of bladder cancer cells *in vitro* [121]. Therefore, understanding the role of macrophages in tumour progression provides valuable ideas for anti-tumour therapy.

### 1.2.6 Macrophage phagocytosis

Phagocytosis is the main function of macrophages in defence against pathogens and contributes to innate and adaptive immune responses [122]. The process of phagocytosis includes receptor recognition, actin polymerization, phagosome maturation and degradation of pathogens [122]. Macrophages have 'pattern recognition receptors' (PRRs) to sense the 'pathogen-associated molecular patterns' (PAMPs) in microbial pathogens, including bacteria, parasites and virus [123]. Macrophage phagocytosis related PRRs include mannose receptor, complement receptors (CR) and the Fc family of receptors (FcR) [124], these mediate phagocytosis through binding non-opsonized targets or opsonic molecule coated targets [125].

The mannose receptor binds and internalizes non-opsonized bacterial and fungal pathogens [119] by recognizing mannose-rich glycoconjugates [126]. The mannose receptor mediated non-opsonized phagocytosis is important in the early immune response before antibodies are synthesized [122]. Opsonized phagocytosis with antibody or complement is more efficient than non-opsonized phagocytosis in eliminating pathogens [127]. The mannose receptor mediated phagocytosis leads to the production of reactive oxygen intermediates and pro-inflammatory cytokines, including TNF [127], IL-6 and IL-1β [128]. The members of the complement receptor family (CR1, CR3, CR4) recognize complement coated microbes [129]. CR1 is mainly involved in binding C3b, C3bi and C4b, while CR3 and CR4 bind C3bi specifically [122]. Unlike the mannose receptor mediated phagocytosis, CR-mediated phagocytosis does not release the pro-inflammatory cytokines or reactive oxygen [130].

FcRs recognize the Fc fragment of immunoglobulin- coated pathogens and they are classified into activating receptors (FcγRI, FcγRIIA, FcγRIII) and inhibitory receptors (FcγRIIB) [131]. The activating FcRs have immunoreceptor tyrosine-based activation motifs (ITAMs) to activate downstream signal cascades. The inhibitory FcRs contain receptor tyrosine-based inhibition motifs (ITIMs) which dampen phagocytosis [131]. After initiation of phagocytosis, the tyrosine kinase Src phosphorylates ITAMs of FcγR to phosphorylate ITAMs. This recruits

and activates the tyrosine kinase Syk [132]. The impaired macrophage phagocytosis in Syk knockout mice indicates the essential role of Syk in phagocytosis [132]. The activation of Syk results in a series of downstream events such as rearrangement of actin and production of inflammatory mediators [122]. On the other hand, ITIMs mediate inhibition of phagocytosis which is important to avoid excessive phagocytosis. The phosphorylated ITIMs activate SH2 domain-containing inositol 5'-phosphatase (SHIP) and then dampen subsequent signalling in phagocytosis inhibition [132]. Some downstream kinases are activated to stimulate the actin polymerization and induce phagosome formation. These kinases include PI-3 kinase, the rho family of GTPases, and protein kinase C (PKC) [122]. The PI-3 kinase inhibitor wortmannin or Ly294002 impairs Fc-receptor mediated phagocytosis by preventing the closure of phagosomes [133]. Members of Rho family of GTPases regulate Fc-receptor mediated phagocytosis by regulating cell spread, cell membrane ruffling and formation of stress fibres [134].

After the recognition of FcRs, pathogens are formed into membrane wrapped phagosomes and phagocytosed by macrophages [125]. The phagosomes fuse with lysosomes to form the phagolysosome [125], which degrade the pathogens into small particles in the acidic environment [125]. The V-ATPase and GTPases (Rab5, Rab7) are associated with phagosome maturation [122]. V-ATPase establishes acidification by pumping protons to generate the acidification of phagolysosomes [135]. Rab5 induces membrane fusion of phagosome and endosomes in macrophages [136]. The overexpression of active Rab5 leads to increased killing ability of intracellular parasites [136]. Rab7 is required for late endosome formation as blocking of Rab7 leads to the impairment of phagolysosome formation and acidification [137]. The process of phagocytosis requires coordination of such events as receptor recognition, actin polarization, membrane trafficking, microbial killing and release of inflammatory cytokines, all of which regulate appropriate immune responses.

### 1.2.7 The inflammasome

In response to intracellular infection, the activation of inflammasome is the main inflammatory response to eliminate microbial invasion. Inflammasome is a group of protein platforms that sense intracellular stimulations and release of pro-inflammatory cytokines IL-1β and IL-18 [138]. The receptors related to inflammasome activation belong to pattern recognition receptors include toll-like receptors (TLRs), node-like receptors (NLRs) and RIG- like

helicases [139]. TLRs have 10 members in humans and 13 members in mice [140]. TLR2 binds a variety of bacterial products such as lipopeptide and peptidoglycan. TLR3 recognizes double-stranded RNA (dsRNA) and TLR4 recognizes the Gram-negative component LPS. The bacterial and viral CpG motifs are recognized by TLR9 [140]. NLRs have 23 members in human and over 34 members in mice [138]. They are composed of a N- terminal effector region caspase recruitment domain (CARD), C-terminal leucine-rich repeats (LRRs) and pyrin domain (PYD) acidic domain, a critical domain for sensing stimulus [141]. Some members of NLRs including NLRP1, NLRP3, NLRC4 are related to the formation of inflammasome, which is essential in host defense against infection [139].

#### 1.2.7.1 The NLRP3 inflammasome

After phagocytosis, the lysosomal disruption with the release of lysosomal contents in the cytoplasm, might be a common pathway that results in the activation of NLRP3 inflammasome [142]. It is supported by the observation that the inhibition of lysosomal disruption leads to the inhibition of inflammasome activation [142]. NLRP3 is a member of NLR proteins and it is the most fully characterized inflammasome. NLRP3 inflmmasome consists of NLRP3 scaffold, ASC adaptor, and caspase-1. It is activated upon the infection of pathogens and self-derived molecules. The pathogens include a range of microorganisms (bacteria, fungi), microbial stimuli (LPS, bacterial RNA, dsRNA analogy poly I:C [143]). Self-derived molecules include ATP from injury cells, elevated excellular glucose from metabolic stress, and monosodium urate (MSU) crystals from gout [141]. NLRP3 inflammasome is also activated by environmental irritants, such as UVB irradiation, silica, and asbestos [138].

Two steps are required in NLRP3 inflammasome activation. The first step is primed by transcriptional activation of NLRP3 and pro-IL-1β through NF-κB signalling activation [144]. After priming, the NLRP3 inflammasome is activated by ATP or bacterial toxins. Caspase-1 is then activated and leads to IL-1β maturation and secretion by cleaving pro-IL-1β [138]. Caspase-1 is the active form of pro-caspase-1, and the caspase-1 deficient mice have impaired IL-1β release, suggesting the essential role in IL-1β maturation [145]. The activation of caspase-1 is related to a form of cell death termed pyroptosis, which has the features of both apoptosis and necrosis [146]. NLRP3 inflammasome activation involves $K^+$ efflux, which is induced by pore formation in the cell membrane, reactive oxygen species (ROS) production and mitochondria damage [138]. ATP stimulation induced low intracellular $K^+$ concentration is critical for NLRP3 inflammasome activation [147]. Moreover, incubation of the cells with

high extracellular $K^+$ concentration abolishes NLRP3 inflammasome activation [147]. ATP induced ion channel purinergic ATP-gated P2X7 receptor (P2X7R) is required in NLRP3 inflammasome activation [148]. The activation of P2X7R leads to the collapse of normal ionic gradients, allowing for the release of intracellular $K^+$ [148]. Moreover, the production of ROS has been suggested to be related to NLRP3 inflammasome activation. Inhibitors of ROS generation leads to the impaired IL-1β production, suggesting the requirement of ROS in inflammasome activation [149]. Mitochondria, but not NADPH oxidase, is the essential source of ROS required for inflammasome activation [150]. The inhibition of mitochondrial ROS scavenger Mito-TEMPO inhibits the activation of NLRP3 inflammasome [150]. The ligand for NLPR3 inflammasome might be from ROS released from the mitochondria, but requires further study. NLRP3 inflammasome senses mitochondrial dysfunction and initiates immune responses in the cellular stress.

#### 1.2.7.2 The NLRC4 inflammasome

NLRC4 is another member in NLR family involved in inflammasome [151]. NLRC4 inflammasome is composed by NLRC4, ASC, caspase-1 and the BIR-domain-containing protein NAIP5 [152]. NLRC4 inflammasome recognizes flagellin from bacteria such as *L. monocytogenes*, *Salmonella* and *Pseudomonas* species [153]. Flagellin of bacteria binds NLRC4 and leads to the activation of NLRC4 inflammasome, thus results in the release of IL-1β [151]. The activation of NLRC4 inflammasome is inhibited when macrophages are infected with flagellin deficient *Salmonella* strains [154]. NLRC4 and NAIP5 appear to bind a similar region of flagellin in bacteria [155]. NLRC4 is required in NAIP5 induced caspase-1 activation, but NAIP5 is independent in the activation of caspase-1 by NLRC4 [155]. Interestingly, the flagellate free bacteria *Shigella flexneri* also activates NLRC4 inflammasome [156]. The activation of NLRC4 inflammasome can also be blocked by bacteria, such as *Mycobacterium tuberculosis [157]*. These data suggest the other molecules beside flagellin also can be recognized by NLRC4 inflammasome, which remains to be uncovered.

#### 1.2.7.3 The AIM2 inflammasome

The cytosolic double-stranded DNA from bacteria, virus and host are recognized by the absent in melanoma-2 (AIM2) inflammasome. AIM2 belongs to the HIN200 protein family which shares a repeat of 200 amino acids [158]. AIM2 inflammasome consists of AIM2, ASC, and caspase-1 [153]. AIM2 binds dsDNA via the C-terminal HIN200 domain and activates

caspase-1 by recruiting the adaptor ASC [159]. Similar to other inflammasome, the activation of caspase-1 results in the maturation and secretion of IL-1β [159]. Ligands activate AIM2 inflammasome are cytosolic dsDNA from bacteria, virus or host. AIM2 inflammasome is the only inflammasome directly binds to the dsDNA that activates inflammasome formation [152]. As a sensor of cytoplasmic DNA, AIM2 inflammasome is an attractive target for treatment in dsDNA related autoimmune disease, including Systemic Lupus Erythematosis (SLE) [153]. AIM2 inflammasome triggers pyroptosis which is a caspase-1 dependent cell death [151]. Pyroptosis is often observed in cytosolic pathogen infection, and it is immunologically 'silent' [151]. In addition to the AIM2 inflammasome activation, the presence of cytosolic DNA is sensed by cGAS/STING [160]. Cyclic-GMP-AMP (cGAMP) synthase (cGAS) binds DNA and synthesizes cGAMP which subsequently binds and activates STING in endoplasmic reticulum membrane [161]. The activation of STING triggers the activation of transcription factor NF-κB and IRF3 [160]. Cytosolic RNA is detected by RIG-I like receptors to produce cytokines such as IFNβ [161].

#### 1.2.7.4 Inflammasome and human disease

IL-1β is a key pro-inflammatory cytokine and the elevated production of IL-1β is related to many diseases, including gout, type II diabetes (T2D) and Alzheimer's disease [138]. The metabolic disturbances of gout is associated with elevated deposition of monosodium urate (MSU) crystals in joints [162]. MSU activates NLRP3 inflammasome activation *in vitro* and MSU-dependent neutrophils infiltration requires the activation of IL-1R, ASC and caspase-1 in *vivo* [162]. Increased IL-1β secretion is involved in T2D development and it drives the pancreatic islets β cell destruction and glucose-induced insulin secretion [163]. High extracellular glucose also triggers NLRP3 inflammasome and IL-1β secretion [164]. Mutations of NLRP3 have been identified as the cause of some rare inflammatory diseases, including the familial cold auto inflammatory Muckle-Wells syndrome and neonatal onset multi-systemic inflammatory disease [142]. The NLRP3 mutation leads to consistent activation of NLRP3 inflammasome with increased caspase-1 activation and IL-1β release. It has been reported that monocytes from the patients with NLRP3 mutations produce IL-1β spontaneously [165]. Anti-IL-1β therapy using IL-1 receptor antagonist has been used to reverse clinical symptoms [166]. The Anakinra which is an antagonist of IL-1RA has been applied in clinical trials [167]. Additionally, the high affinity for IL-1, IL-1Trap, and IL-1β neutralized monoclonal antibodies are available to the clinical trials for a wider range of diseases [167]. The future research of

inflammasome associated mechanisms will provide new approaches to autoimmune disease treatments.

### 1.3 The intracellular bacterium- *Listeria monocytogenes*

*Listeria monocytogenes* (*L. monocytogenes*) was first identified in 1926, as is a gram-positive bacterium which causes lethal disease in a rabbit colony [168]. *L. monocytogenes* is found in water, soil and uncooked meat and cheeses [169]. *L. monocytogenes* can cause serious infections in immunocompromised individuals, neonates and pregnant women [169]. Pregnant women develop chorioamnionitis and septic abortion with the infection of *L. monocytogenes* [170]. Gastroenteritis can be caused by the exposure to *L. monocytogenes* by ingesting incompletely cooked meats and unpasteurized dairy products [170]. Therefore, *L. monocytogenes* is a permanent pathogen in caring for pregnant women, neonates, and immunocompromised individuals. As *L. monocytogenes* is one of the best characterized and easily manipulated bacterial pathogens. It has been widely used in the research of interface between the mammalian immune system and a pathogenic microorganism [169].

#### 1.3.1 Life cycle of *L. monocytogenes* in macrophages

*L. monocytogenes* infects various cells including macrophages, epithelial cells [171], fibroblasts and neurons [172]. Macrophages are the main effector cells for clearing *L. monocytogenes* infection [173]. *L. monocytogenes* are phagocytosed by macrophages in membrane-coated phagosomes [174]. *L. monocytogenes* then escapes from the acidic environment of the phagosome into the cytoplasm by lysing the membrane of the phagosome. After escape, *L. monocytogenes* reproduces rapidly and rearranges the actin to move in the cytoplasm by forming pseudopods [174]. Neighbouring cells then engulf the pseudopods to form double membrane vacuoles with *L. monocytogenes* and start the new cycle of infection [174]. The product of the hemolysin (*hly*) genes in *L. monocytogenes*, listeriolysin O (LLO), is essential for escape from the primary and secondary vacuoles [174]. LLO is a pore-forming toxin belonging to the cytolysin group that is commonly expressed by gram-positive bacteria [175]. The lytic activation of LLO is optimal in acidic pH and leads to *L. monocytogenes* escape from phagosomes [176]. The LLO-dependent vacuole fusion with lysosomes therefore inhibits *L. monocytogenes* degradation in the acidic environment. LLO-deficient *L. monocytogenes* escapes inefficiently compared with wild type *L. monocytogenes* [177].

After *L. monocytogenes* escape from the phagosome, the cell surface protein ActA is anchored to the membrane of mitochondria and modulates the movement of *L. monocytogenes* by rearranging the actin in the cytoplasm [178]. ActA is the product of gene *actA* and the *actA* mutant of *L. monocytogenes* leads to inhibition of intracellular movement [179]. ActA is a surface protein has 639 amino acids with a transmembrane motif at its carboxyl-terminal domain. The domain containing four proline-rich repeats triggers the *Listeria* actin-based motility [180]. The region of ActA (amino acids 31–263) is crucial in inducing bacterial movement, through activating Arp2/3, inducing actin polymerization and generating the array of actin filaments [181].

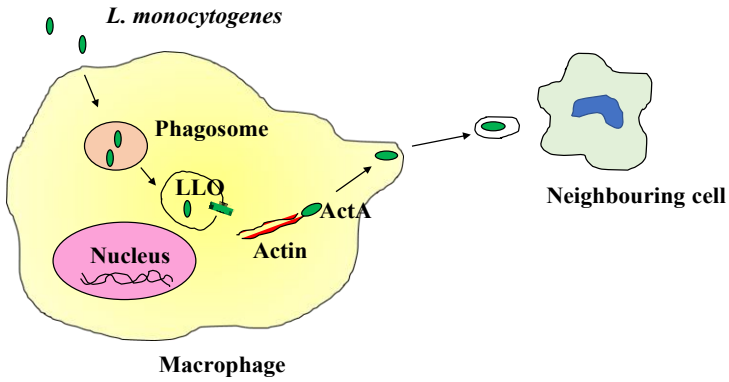


**Figure 1.4: Macrophage phagocytosis of *L. monocytogenes*.** *L. monocytogenes* is phagocytosed by macrophages in membrane formed phagosome. *L. monocytogenes* then expresses LLO to lyse the membrane and escape from the vacuole. In the cytoplasm, *L. monocytogenes* reproduces rapidly and expresses ActA to rearrange the actin to move into the cytoplasm. Sequentially, the released *L. monocytogenes* infects healthy neighbouring cells to start the new cycle of infection. Adapted from [4].

### 1.3.2 Macrophage responses during *L. monocytogenes* infection

Monocytes are essential phagocytes in host defence against *L. monocytogenes* infection. Ly6C$^{hi}$ monocytes, recruited from bone marrow, differentiate into TNFα and iNOS producing dendritic cells (TipDCs) [69]. The chemokine CCR2 is required for Ly6C$^{hi}$ monocyte recruitment into the infected sites [182]. CCR2 deficiency leads to reduced Ly6C$^{hi}$ monocyte infiltration to spleen and increased susceptibility to *L. monocytogenes* infection [182]. IFNγ produced by NK cells is important for maturation of Ly6C$^{hi}$ monocytes [84]. Ly6C$^{low}$ monocytes are recruited in the early stages of *L. monocytogenes* infection. They transiently secrete TNF then display anti-inflammatory phenotypes with up-regulated expression of Fizz1

and CD206 [71]. The intracellular adaptive protein MyD88 is required for host defence against *L. monocytogenes* infection [183].

*L. monocytogenes* has a range of TLR ligands such as flagellin and peptidogylcan which can be recognized by macrophages [173]. TLRs are required for inflammatory cytokine production and host response to *L. monocytogenes* infection [184]. For instance, *L. monocytogenes* infected macrophages produce high amounts of TNF and IL-12 through the activation of the TLRs. Mice deficient in the TLR adaptor molecule MyD88 are susceptible to *L. monocytogenes* infection and are unable to produce TNF and IL-12 in response to TLR engagement [184]. Macrophages also produce chemokines such as CCL-2, and CCL-7 to recruit inflammatory monocytes into the infected tissue [184]. Activated macrophages are essential for sensing and eliminating *L. monocytogenes* infection. The activated macrophages are stimulated by IFNγ in combination with LPS or TNF. The high levels of nitric oxide produced by activated macrophages are bactericidal and effective in killing *L. monocytogenes* as iNOS deficient mice are highly susceptible to *L. monocytogenes* infection [185]. However, the enhanced production of NO is a double-edged sword in *L. monocytogenes* elimination [186]. *L. monocytogenes* takes advantage of the high NO concentration to promote their spread during infection. The iNOS inhibitor W1400 significantly reduces *L. monocytogenes* spread in the hosts [186]. Activated macrophages inhibit phagosome escape of *L. monocytogenes* through reactive oxygen intermediates (ROI) and reactive nitrogen intermediates (RNI) [97]. The inhibitors of ROI and RNI block *L. monocytogenes* vacuolar escape in macrophages [97]. The production of ROI and RNI are co-localized with *L. monocytogenes*, suggesting the direct microbial activities in the individual phagosomes of macrophages.

### 1.3.3 Inflammasome activation in *L. monocytogenes* infection

*L. monocytogenes* infection is also sensed by inflammasomes which is crucial for macrophage responses to infection through the production of IL-1β and IL-18 [187]. *L. monocytogenes* activated inflammasomes include NLRP3, NLRC4 and AIM2 [187]. NLRP3 inflammasomes are activated following *L. monocytogenes* infection and NLRP3 deficiency in macrophages leads to impaired caspase-1 activation and IL-1β release in response to *L. monocytogenes* infection [188]. Furthermore, inflammasome activation depends on the cytosolic invasion of *L. monocytogenes* as IL-1β cannot be secreted by macrophages when infected with the LLO deficient *L. monocytogenes* [189], consistent with the essential role of cytosolic signaling in

host responses to *L. monocytogenes* infection. In addition to the IL-1β release, *L. monocytogenes* infection also leads to caspase-1 dependent cell death [189]. NLRC4 inflammasome senses flagellin in *L. monocytogenes* and leads to caspase-1, IL-1β activation [190]. AIM2 inflammasome senses *L. monocytogenes* by recognizing DNA of *L. monocytogenes* in the cytosol [159]. The bacterial DNA is released after *L. monocytogenes* escape from phagolysosome [158]. Knockdown of AIM2 in macrophages leads to impaired caspase-1 activation, IL-1β release, and cell death after infection of *L. monocytogenes* [159].

### 1.4 Devil facial tumour disease

The Tasmanian devil (*Sarcophilus harissii*) is the largest marsupial carnivore in the world, since the extinction of Tasmanian tiger (*Thylacinus cyanocephalus*) in 1936 [191]. Devils are nocturnal scavengers that feed on carrion and sometimes hunt for birds and small mammals [192]. They are medium sized and weigh between 4.5 kg and 13.0 kg [191]. The jaws and teeth of devils are extremely powerful which enable them to devour their prey [192]. This species is threatened by the fatal disease, devil facial tumour disease (DFTD). DFTD was first recorded in 1996 and has been the main factor responsible for the decline of devil population [192]. Consequently, devils are listed as endangered as a result of the DFTD epidemic. The extinction of the Tasmanian devil would be crucial in the ecosystem. It is risky to feral cats and potentially foxes, which would be the disastrous consequences for native species [193].

#### 1.4.1 Pathology and origins of DFTD

DFTD is transmitted as an allograft. DFTD cells are the infectious agent and transmitted between devils by biting [194]. The biting in Tasmanian devils is a common interaction in mating seasons and feeding [195], suggesting that biting is the key means of disease transmission. DFTD appears as firm, spherical nodules, or ulcerate tumours located on the face, neck and head [192]. The face and neck are the primary locations of DFTD and the tumours often show necrosis, bacterial contamination, and metastasis to other organs [196]. DFTD was derived from a female devil in the northeast Tasmania in 1996 [197]. Research shows that DFTD originated from a Schwann cell [198]. The Schwann cell myelin protein, periaxin (PRX) is expressed in primary DFTD tumours, and DFTD cultured cells, suggesting its utility as a marker for DFTD [198]. There are several other markers such as S100, nerve growth factor receptor and myelin basic protein that identify DFTD cells, albeit inconsistently [196]. The clonal origin of DFTD has been confirmed by sequencing of the multiple microsatellite loci of tumour and matched devil hosts. All the tumours were identical at MHC and multiple

microsatellite loci, and differed from their hosts [199]. In contrast with the female devil derived DFTD (DFT1), the second transmissible cancer, DFT2 was identified in 2015 [200]. DFT2 has distinct histological and genetic phenotypes from the DFT1. It carries a Y chromosome in comparison of the two X chromosomes, different alleles to its hosts at MHC loci and microsatellite [200]. As a transmissible allograft tumour, there are several possible explanations for the establishment of DFTD in the devil population: incompetent immune system of devils; low genetic especially at the MHC loci; or tumour escape from the host's immune system.

### 1.4.2 The devil's immune system

Tasmania devils are scavengers and are exposed to a wide range of bacteria and parasites due to their diet and biting behaviour. Little evidence shows that wild devils succumb to disease of bacteria and parasites. Similarly, research has demonstrated functional immune responses [201]. Thus, it is assumed that devils have a fully competent innate immune system which provides primary protection against these pathogens [196].

Studies on basic histological and immunology function assay suggest that devils have a competent immune system [202]. Devil tissue architecture and distribution of the immune cells were analysed by using CD3, CD79b and MHCII antibodies [202]. The thymus, spleen and peripheral lymph nodes have similar structure to mammals and other marsupials. This study indicated that devils have the immune system competent required for immune responses [202]. Neutrophils isolated from peripheral blood of devils exhibit the ability to phagocytose *E. coli* [203]. The respiratory burst in neutrophils was identified by the nitro blue tetrazolium assay, suggesting the active oxygen dependent pathway in phagocytosis of bacteria [203]. NK-like cells from devil peripheral blood exhibit rapid cytotoxic responses in the presence of antibody [204]. The peripheral blood mononuclear cells from Tasmanian devils proliferate in response to PHA, PMN and Con A stimulation [203]. In response to horse red blood cells (HRBC) injection, devils showed evidence of antibody production, suggesting the Tasmanian devils are capable of humoral immune responses [205]. Following immunization with xenogeneic human erythroleukemia line K562 cells, devils produced antibodies and cytotoxic responses [204].

### 1.4.3 Low genetic diversity of devils

Since devil facial tumours are allografts, the devil immune system should recognize the DFTD cells as foreign pathogens. Why do DFTD cells survive as an allograft? The low diversity of

MHC class I of the species was put forward as one explanation [199]. From a study in 1985, South African cheetah (Acinonyx jubatus) were shown to have extremely low genetic diversity including Major Histocompatibility Complex (MHC) [206]. The acceptance of skin grafts between unrelated cheetahs indicated the species vulnerability in the cheetah [206]. The lack of MHC class I diversity is the cause of the spread of contagious tumour in the Syrian hamster [207]. Similarly, the analysis of whole-genome sequence of Tasmanian devils indicated the moderate genetic diversity [208]. However, skin allografts were rejected 14 days after surgery with extensive T cell infiltration, the low genetic variety at MHC cannot explain the failure of devils to recognise DFTD cells. [209].

DFTD cells in vivo and in vitro have been shown to have low expression of MHC class I, suggesting the depletion of antigen- processing pathway [199]. Analyses of MHC transcript show that DFTD cells have functional MHC genes. [199]. Treatment of DFTD cells with histone deacetylase inhibitor trichostatin A (TSA) restored the MHC class I expression [199]. This evidence showed that the absence of expression of MHC I on the cell surface is due to epigenetic modifications rather than structural mutations [199]. Some human tumours also have structural mutations of MHC I genes with decreased MHC I molecule expression which leads to escape from effective T cell responses [210]. Recombinant devil IFNγ treated DFTD cells with increased expression of MHC I, has been used in the anti-DFTD vaccine protocol. On the other hand, NK cells would be expected to be effector cells to DFTD cells in response to the lack of MHC I expression. However, the DFTD cells can't be recognized by devil NK cells [211], further investigations are required to find the mechanism involved.

### 1.4.4 Immunosuppressive Cytokines

The immunosuppressive cytokine such TGF-β and IL-10 are released from tumour cells in the micro-environment favouring tumour progression and host immune response suppression [196]. TGF-β regulates immune responses by modulating T cell activation, differentiation and proliferation [212]. IL-10 inhibits T cells activation by inhibitory effects on macrophages and DCs [213]. In the analysis of TGF-β and IL-10 transcriptome from DFTD biopsies, there was no significant upregulation in tumour tissues [214]. Detection of the expressions of TGF-β and IL-10 at protein level would be valuable to quantify these cytokines. Understanding the mechanism of DFTD immune escape is important in development of vaccines to protect Tasmanian devils in the wild.

### 1.4.5 Macrophages and devils

Macrophages play an important role in tumour development. Immunohistochemical analysis has shown macrophages in Devil spleen. These macrophages are in irregular shapes, with large phagosomes and numerous mitochondria in the cytoplasm [215]. The lack of devil-specific reagents such as devil derived M-CSF or GM-CSF has been the main obstacle in culturing devil macrophages *in vitro*. Devil specific antibodies for immunohistochemistry and flow cytometry are not available for the investigation of macrophage activity in DFTD.

## 1.5 Final remarks

TNF is an important pro-inflammatory cytokine in response to infection. TNF is mainly produced by macrophages and it activates macrophages with high efficiency in bacterial elimination. There is evidence of TNF in inhibition of AAM differentiation in parasitic infection [35] and tumour model [34]. In this study we investigate the common activity of TNF in macrophage differentiation in another infection model, *L. monocytogenes*. Splenic and peritoneal macrophages have been used to determine the role of TNF in AAM differentiation during *L. monocytogenes* infection.

Phagocytosis is the main function in macrophage defence against pathogens. The effects of TNF in macrophage phagocytosis under different activation status of macrophages is unclear. Consequently, we investigated role of TNF in macrophage phagocytosis of tumour cells under different activation conditions. The tumour cells used in this study were Devil Facial Tumour Disease (DFTD) cells. These tumour cells were selected to determine if DFTD cells could be phagocytosed. The understanding of macrophage phagocytosis in DFTD cells will improve the knowledge of DFTD immune escape mechanisms.

## 1.6 Aims of thesis

The therapeutic modulation of TNF has been used widely to treat chronic inflammatory diseases. The TNF antagonists such as Infliximab® (Centocor), Etanercept® (Amgen/Wyeth), Certolizumab®, Golimumab® [216] have been showing good therapeutic effect in the treatment of Rheumatoid arthritis, Crohn's disease, Ankylosing spondylitis and Psoriasis [217, 218]. However, it has been implicated that anti-TNF treatment can cause the recrudescence of existing latent infections, such as tuberculosis [218] and leishmaniasis [219]. The roles and mechanisms of TNF in mediating protection or pathology during infection are still unclear in

some details and need to be investigated. Therefore, a comprehensive investigation of the role of TNF in innate immune responses to intracellular pathogens such as *L. monocytogenes* will provide a better understanding of potential consequences of using TNF antagonists to treat chronic inflammatory diseases.

Macrophages are important innate immune cells in regulating immune response to pathogens. TNF is mainly produced by macrophages and activates macrophages with higher ability in elimination of pathogens. TNF activates macrophages into CAMs in concert with IFNγ. Additionally, TNF has been reported to inhibit AAMs polarization in the condition of tumour [34] and *L. major* infection [35]. Therefore, role of TNF in macrophage polarization during bacterial infection is worth to investigate. Macrophages release pro-inflammatory cytokine IL-1β during bacterial infection by inflammasome activation. TNF in regulating macrophage releasing IL-1β during bacterial infection is interesting to investigate. Macrophage phagocytose pathogens and consequently eliminate them and regulate immune responses. TNF in regulating macrophage phagocytosis of bacteria and the other target such as DFTD cells is investigated.

Thus, the aims of my thesis are as followed:

Aim 1: To investigate the role of TNF in splenic monocyte differentiation following *L. monocytogenes* infection

Aim 2: To examine TNF regulation of peritoneal monocyte differentiation upon *L. monocytogenes* infection

Aim 3: To investigate the role of TNF in macrophage phagocytosis during anti-bacterial and anti-tumour defence

# Chapter 2

## Chapter 2 Materials and methods

**Table 2.1 Commonly used reagents**

| Reagent | Supplier | Catalogue number |
|---|---|---|
| 2-Mercaptotoethanol | Gibco | 21985023 |
| Acetic acid glacial | BDH AnalaR | Prod 10001 |
| Acetone | Sigma-Aldrich | 270725-2L |
| Adenosine 5′-triphosphate (ATP) | Sigma-Aldrich | A2383-1G |
| Bovine serum albumin (BSA) | Sigma-Aldrich | A7906-100G |
| Brain Heart Infusion Broth (BHI) | BD bioscience | 237500 |
| Cell count beads | eBioscience | 01-1234-42 |
| Collagenase IV | Sigma-Aldrich | M9445 |
| DAPI | Sigma-Aldrich | D9542 |
| Ethanol | Sigma-Aldrich | E7023-500ML |
| Ethylenediaminetetraacetic acid (EDTA) | Sigma-Aldrich | EDS-500G |
| Fetal calf serum (FCS) | Gibco | 10099-141 |
| Fluorescent mounting medium | Dako Cytomation | S3023 |
| Gelatin | Sigma-Aldrich | G1890-100G |
| Gentamicin | Invitrogen | 15750060 |
| Glycerol | Sigma-Aldrich | G6279-500ML |
| Halt® protease inhibitor | Thermo Fisher | 78430 |
| HEPES | Sigma-Aldrich | H3375-100G |
| Interferon γ (IFNγ) | Peprotech | 315-05 |
| Interleukin-4 (IL-4) | Peprotech | 214-14 |
| L-Glutamine | Gibco | 25030081 |
| Lipopolysaccharide (LPS 055:B5) | Sigma-Aldrich | L6529-1MG |
| Milli-Q® water | Biocel | QGARDOORI |
| N-(1-Naphthyl) ethylenediamine dihydrochloride($C_{10}H_7NHCH_2CH_2NH_2 \cdot 2HCl$) | Sigma-Aldrich | N9125-100G |
| Non-essential amino acids | Gibco | 11140050 |
| Penicillin/streptomycin | Gibco | 15140122 |
| Phosphoric acid ($H_3PO_4$) | Sigma-Aldrich | 438081-500ML |
| Potassium chloride (KCl) | Sigma-Aldrich | P3911-500G |

| Reagent | Supplier | Catalogue number |
|---|---|---|
| Potassium dihydrogen phosphate ($KH_2PO_4$) | Sigma-Aldrich | P9791-500G |
| Proteinase k | Sigma-Aldrich | P6556-5MG |
| Rat serum | Sigma-Aldrich | R9759-10ml |
| RPMI 1640 medium | Invitrogen | 11875093 |
| Sodium azide ($NaN_3$) | Sigma-Aldrich | S2002-100G |
| Sodium chloride (NaCl) | Sigma-Aldrich | S5886-500G |
| Sodium nitrite ($NaNO_2$) | Sigma-Aldrich | 237213-500G |
| Sodium phosphate dibasic ($Na_2HPO_4$) | Sigma-Aldrich | 30435-500G |
| Sodium pyruvate | Gibco | 11360070 |
| Sulfanilamide ($H_2NC_6H_4SO_2NH_2$) | Sigma-Aldrich | S9251-100G |
| Thioglycollate | BD bioscience | 211716 |
| TMB | ThermoFisher | N301 |
| Tris base | Sigma-Aldrich | T1378-1KG |
| Triton X-100 | Sigma-Aldrich | X100-100ML |

**Table 2.2 Commonly used disposables**

| Disposables | Supplier | Catalogue number |
|---|---|---|
| 1 mL Syringe | Terumo | 20650240 |
| 10 μl Tips | VWR | LAC23584 |
| 10 ml Syringe | Terumo | 20650240 |
| 1000 μl Tips | VWR | SORELAC1707 |
| 15 ml Centrifuge tube | Corning | CLS430791-500EA |
| 200 μl Tips | VWR | LAC1702 |
| 22x 22 mm Coverslip | Deckglaser | 22 x 22 mm |
| 40 μm cell Strainers | Falcon | 21008-949 |
| 50 ml Centrifuge tube | Corning | CLS430829-500EA |
| CellTrace™ CFSE Cell Proliferation Kit | ThermoFisher | C34554 |

| Disposables | Supplier | Catalogue number |
|---|---|---|
| CellTrace™ Violet Cell Proliferation Kit | ThermoFisher | C34557 |
| Elisa 96-well plate | Sarstedt | 82.1583.200 |
| Eppendorf tube | Eppendorf | 80-1500 |
| Flat-bottomed 96-well microplate | Corning | NUN167008 |
| Fluorescent mounting medium | DakoCytomation | S3023 |
| FOXP3 fix/perm kit | eBioscience | 00-5523-00 |
| iScript™ gDNA Clear cDNA Synthesis Kit | Bio-Rad | 1725034 |
| iScript™ gDNA Synthesis Kit | Bio-Rad | 172-5035 |
| Light microscope | Leica | DM2500 |
| Live/dead fixable Aqua dead cell stain kit | ThermoFisher | L34957 |
| Microscope slide | Livingstone | 7105-U |
| Needle 21G | Terumo | 20650030 |
| Needle 26G | Terumo | 20650050 |
| NucleoSpin® RNA XS kit | Macherey-Nagel | 740902.50 |
| NueleoSpin® RNA XS kit | Macherey Nagel | 740902.50 |
| NuPAGETM gel | Life technologies | NP0322BOX |
| Protein broad range standard | Bio-Rad | 161-0317 |
| ReliaPrep™ RNA Cell Miniprep System | Promega | Z6011 |
| ReliaPrep™ RNA Cell Miniprep System | Promega | Z6011 |
| Round-bottomed 96-well microplate | Corning | CLS3799-50EA |
| SsoAdvanced™ Universal SYBR® Green Mix | Bio-Rad | 172-5271 |
| Streptavidin HRP | BD | 554066 |

**Table 2.3 Commonly used equipment**

| **Equipment** | **Supplier** |
|---|---|
| – 80 °C Freezer | Sanyo |
| Autoclave | Atherton |
| Bench top centrifuge | Beckman Coulter |
| Bench top microcentrifuge | Thermo scientific |
| BX50 Fluorescent microscope | Olympus |
| Canto II flow cytometer | BD biosciences |
| Class II biological safety cabinet | Gelman Sciences |
| Confocal microscope | Nikon |
| Cyan ADP flow cytometry | Beckman Coulter |
| iBlot™ 2 dry blotting system | Invitrogen |
| Incubator | Sanyo |
| Light microscope | Olympus |
| Lightcycler 480 qPCR instrument | Roche |
| Microplate reader | Bio-Rad |
| MoFlo Astrios cell sorter | Beckman Coulter |
| pH meter | inoLab |
| Platform mixer | Ratek Instruments |
| Water bath | Thermoline |

## 2.4 Solutions

### 2.4.1 0.5 M EDTA stock

EDTA 46.53 g

EDTA was dissolved in 200 ml Milli-Q® water, the pH was adjusted to 8.0 with NaOH, and the volume was adjusted to 250 ml with Milli-Q® water.

### 2.4.2 0.5% Gelatine

| | |
|---|---|
| Gelatine | 0.5 g |

Gelatine was dissolved in 100 ml PBS and stored at 4 °C after autoclave.

### 2.4.3. 50% Glycerol stock

| | |
|---|---|
| Glycerol | 20 ml |
| PBS | 20 ml |

Under sterile condition, glycerol was mixed with PBS. The solution was stored at 4 ºC.

### 2.4.4. 10x Phosphate buffered saline (PBS)

| | |
|---|---|
| NaCl | 80.0 g |
| KCl | 2.0 g |
| $Na_2HPO_4$ | 14.4 g |
| $KH_2PO_4$ | 2.4 g |
| Milli-Q® water | 1000 ml |

Using a magnetic stirrer, the reagents were dissolved in Milli-Q® water. The volume was adjusted to 1000 ml. The solution was diluted in 10 times before use.

### 2.4.5. 3x Western blot sample buffer (For 500 ml)

| | |
|---|---|
| 187.5 mM Tris–HCl (pH 6.8) | 178 ml 0.5-M Tris–HCl pH 6.8 stock |
| 6% SDS | 150 ml 20% SDS stock solution |
| 0.03% Phenol Red | 150 mg |
| 30% Glycerol | 172 ml 87% Glycerol stock solution |

The reagents were dissolved in Milli-Q® water and stored at 4 ºC. The solution was diluted in 3 times with Milli-Q® water before use. 500 µM DTT was added before freezing samples.

### 2.4.6. BHI broth media

| | |
|---|---|
| BHI | 3.7 g |

BHI powder was dissolved in 100 ml $dH_2O$. The medium was kept at 4 ºC after autoclave.

### 2.4.7. DFTD complete culture medium

| | |
|---|---|
| RPMI 1640 medium | 500 ml |

| | |
|---|---|
| Heat inactivated FCS | 50 ml |
| L-glutamine | 5 ml |
| Penicillin/streptomycin | 5 ml |

Under sterile condition, the RPMI-1640 medium was supplemented with the above solution and mixed by inversion. The solution was stored at 4 ºC.

### 2.4.8. ELISA coating buffer

| | |
|---|---|
| $NaHCO_3$ | 4.2 g |
| $Na_2CO_3$ | 1.8 g |

The reagents were dissolved in 500 ml $dH_2O$ and pH was adjusted to 9.5. The solution was stored at room temperature.

### 2.4.9. FACS buffer

| | |
|---|---|
| PBS | 1.5 l |
| BSA | 3 g |
| 10% AZ buffer | 3 ml |

The reagents were mixed by inversion, and stored at 4 °C.

### 2.4.10. Freeze L. monocytogenes

| | |
|---|---|
| 50% Glycerol | 100 µl |
| PBS | 400 µl |

*L. monocytogenes* to be frozen was centrifuged at 5,000 rpm for 5 min and the supernatant was discarded. The tube containing *L. monocytogenes* pellet was added in 100 µl 50% Glycerol. The solution was mixed and stocked at -80 ºC.

### 2.4.11. Freezing medium

| | |
|---|---|
| Complete DFTD culture medium | 20 ml |
| DMSO | 20 ml |
| FCS | 60 ml |

The reagents were mixed by inversion, and stored at 4 ºC.

### 2.4.12. Griess assay reagent I

| | |
|---|---|
| 1% Sulphanilamide | 0.5 g |
| 2.5% $H_3PO_4$ | 1.25 ml |

The reagents were dissolved in 50 ml Milli-Q® water. The solution was stored at room temperature and protected from light.

### 2.4.13. Griess assay reagent II

| | |
|---|---|
| $NH_2CH_2CH_2NH_2$. 2HCl | 0.05 g |

The reagent was dissolved in 50 ml Milli-Q® water, stored at room temperature and protected from light.

### 2.4.14. Heat inactivated fetal calf serum (FCS)

FCS was thawed at room temperature and heated for 1 h at 57 °C. Aliquots were stored into sterile 10 ml tubes at -20 ºC.

### 2.4.15. Immunofluorescence blocking buffer

| | |
|---|---|
| BSA 1% (10% BSA stock) | 10 ml |
| Sodium Azide | 100 mg |
| Glycine | 2.25 g |

The reagents were dissolved in 100 ml PBS and the solution was kept at 4 ºC.

### 2.4.16. Macrophage complete medium

| | |
|---|---|
| RPMI 1640 | 500 ml |
| Heat inactivated fatal bovine serum | 50 ml |
| L929 hybridoma supernatant | 50 ml |
| Heat inactivated horse serum | 25 ml |
| L-Glutamine (100x) | 5 ml |
| Non-essential amino acids (100x) | 5 ml |
| Penicillin/streptomycin (100x) | 5 ml |
| Sodium pyruvate (100x) | 5 ml |
| 2-Mercaptotoethanol | 500 µl |

Under sterile condition, the RPMI 1640 medium was supplemented with the above solution and mixed by inversion. The medium was stored at 4 ºC.

### 2.4.17. Mice tail lysis buffer

| | |
|---|---|
| 1 M Tris pH 8 | 10 ml |
| 2 M NaCl | 10 ml |
| 0.5 M EDTA pH 8 | 1 ml |
| 10% SDS | 2 ml |

The reagents were dissolved in 100 ml $dH_2O$, stored at room temperature. The solution was diluted with Proteinase k (10 mg/ml) at 50:1 before use.

### 2.4.18. Macrophage harvesting media

| | |
|---|---|
| PBS | 500 ml |
| BSA | 5 ml 10% stock |
| 2 mM EDTA | 2 ml 500 mM stock |

Under sterile condition, the solution was mixed and kept at 4 ºC.

### 2.4.19. Ponceau staining solution (For 500 ml)

| | |
|---|---|
| 0.05% Ponceau S | 250 mg |
| 3% Trichloroacetic acid | 15 g |

The reagents were dissolved in 500 ml $dH_2O$, and kept at room temperature.

### 2.4.20. Red blood cells lysis buffer

| | |
|---|---|
| 0.17 M Ammonium chloride | 9.0933 g/l |
| 0.02 M HEPES | 4.766 g/l |

The reagents were dissolved in 1000 ml $dH_2O$ and stored at room temperature.

### 2.4.21. Spleen lysis buffer

| | |
|---|---|
| PBS | 2.445 ml |
| 1mM EDTA | 5 µl 500 mM stock |
| 0.05% TritonX 100 | 25 µl 5% TritonX 100 |
| Halt® Protease inhibitor | 25 µl (100x dilute) |

Set up 2.5ml spleen lysis buffer and freeze at -20 ºC.

## 2.5 Methods

### 2.5.1 Animals

The gene-targeted C57BL/6 (B6.TNF$^{-/-}$) mouse strains deficient for TNF were generated on a genetically pure C57BL/6 (B6.WT) background as described [220]. The screening procedure followed the protocols described previously [220]. All animals were housed in pathogen-free conditions. Mice aged 8-16 weeks were used in all experiments following approval of the Animal Ethics Committee of University of Tasmania (UTAS) under the ethics number A13933, A13934 and A13936.

### 2.5.2 Cell culture

#### 2.5.2.1 Bone marrow derived macrophages (BMDMs) culture

BMDMs from B6.WT and B6.TNF$^{-/-}$ mice were generated from pelvic and femoral bones. BMDMs were cultured in macrophage complete medium for 7-9 days.

For the experiment of inflammasome, BMDMs were harvested and were activated with LPS at 100 ng/ml in 96 well plates for 3 hours. After washing with PBS twice, BMDMs were co-cultured with *L. monocytogenes* at Multiplicity of infection (MOI) 5, 10, 20, 40 for 40 min. ATP was added for positive control and KCl was for negative control as described previously [221]. BMDMs were incubated with 10 μg/ml gentamicin to kill extracellular bacteria. Cell culture supernatants were collected after 6 hours' incubation and analysed with ELISA and western blot. Cells were lysed using western blot buffer and kept at -80 °C.

For the experiment of phagocytosis assay, BMDMs were treated with the following substances: 20 ng/ml IFNγ, 100 ng/ml LPS or a combination of IFNγ and LPS for 24 h or 5 ng/ml IL-4 for 48 h. Control macrophages were cultured in medium alone. The macrophages were identified as CD11b$^{+}$F4/80$^{+}$ and the purity of macrophages obtained was always at least 90%.

#### 2.5.2.2 Thioglycollate-elicited peritoneal macrophages collection

Thioglycollate-elicited peritoneal macrophages were prepared from the peritoneal cavity of B6.WT or B6.TNF$^{-/-}$ mice. After 3 days after i.p injection of 3% Brewer's thioglycollate broth at dose of 10 μl/g of mice weight. Cells were then adhered to the bottom of petri dish for 2 h and the adherent cells were harvested. The purity of macrophages was analysed using flow cytometry by staining for CD11b and F4/80. Macrophages were cultured in 24 well-plates with

0.2% gelatine coated 12 mm coverslips at $5x10^4$ macrophages/well. The macrophages were subsequently incubated in fresh medium supplemented with 10% FCS or stimulated with 20 ng/ml IFNγ and 100 ng/ml LPS for 24 h or 5 ng/ml IL-4 for 48 h, and infected with *L. monocytogenes* as indicated.

#### 2.5.2.3 Devil Facial Tumour Disease cell culture

The Devil Facial Tumour Disease cell line, C5065, was established from primary tumour samples [222]. Cells were grown in DFTD complete culture medium and maintained in a humidified 5% $CO_2$ incubator at 35 °C. Phagocytosis assays required co-culturing of mouse macrophages with DFTD cells were maintained at 37 °C.

### 2.5.3 *L. monocytogenes* culture

*L. monocytogenes* strain EGD was grown overnight in brain heart infusion (BHI) broth at 37 °C with shaking. Bacterial suspension was diluted at 1:50 in fresh BHI broth and shaken at 37 °C for 2 h to obtain an OD600 of 0.10. 1 ml bacterial culture has $2x10^8$ *L. monocytogenes*. Bacteria were washed by pelleting and washed with PBS for two times. *L. monocytogenes* were suspended in PBS for infection *in vitro* or *in vivo*.

### 2.5.4 Cell collection from *L. monocytogenes* infected mice

#### 2.5.4.1 Splenocytes and bone marrow cells dissociation

Spleens were dissociated with collagenase V in PBS for 10 min at room temperature. Red blood cells were lysed using ACK lysis buffer. Splenocytes were filtered through 40 μm cell strainers to remove cell debris. Cell pellets were suspended in 10 ml FACS buffer for cell counting. Bone marrow cells were collected by flushing from femurs with ice-cold PBS. Red blood cells were lysed using red blood cells lysis buffer and centrifuged at 500 g for 5 min. Cell pellets were suspended in 10 ml FACS buffer for cell counting.

#### 2.5.4.2 Isolation of peritoneal cavity cells

The peritoneal lavage fluid was collected after the infection of *L. monocytogenes.* Mice were injected with 5 ml ice-cold PBS and the mouse abdomen was massaged gently to allow cells to suspend in the lavage fluid. The lavage fluid was extracted gently and slowly using a 21-G needle. The collected peritoneal fluid was transferred to a 15 ml tube and kept on ice, the

volume of fluid was recorded. Peritoneal cells were centrifuged at 500 g for 5 min and suspended in FACS buffer for cell counting.

### 2.5.5 CFUs assay

Spleen and liver were removed from animals and homogenized in 5ml lysis buffer (PBS with 0.05% Triton X-100). Serial 10-fold dilutions of homogenate were made in 96 well plates. Twenty µl undiluted homogenate was added into 180 µl lysis buffer and mixed thoroughly before starting any dilution. Fifty µl homogenate was spread in BHI agar plate and cultured at 37°C overnight. Colony forming units (CFUs) between 20 and 200 were counted and recorded as CFU/organ. Alternatively, to determine the bacterial load in cells, cells were isolated by flow cytometry and 5000 cells were lysed in 50µl buffer. The homogenate was plated in BHI agar plates and CFU were recorded as CFU/5000 cells.

### 2.5.6 Quantitative real-time PCR

The qPCR primers were designed using Primer-BLAST in PubMed. DNA template was entered, to avoid amplification of contaminating genomic DNA, the option 'Primer must span an exon-exon junction' was selected. Primers with GC content of around 50-60% and primer-dimer were needed to be avoided. A standard curve was used to determine qPCR efficiency. Fivefold dilution series of cDNA were run in duplicate. The slope of a graph where log (dilution factor) is on the x-axis and Ct on the y-axis. Efficiency was calculated using the formula: efficiency = 10^ (-1/slope). The efficiency from 90-110% was preferred.

RNA was extracted using ReliaPrep™ RNA Cell Miniprep System or NucleoSpin® RNA XS kit according to the manufacturer's instructions. The iScript™ gDNA Clear cDNA Synthesis Kit was used to reverse-transcribe total RNA. Quantitative real-time PCR was carried out on the Lightcycler 480 qPCR instrument with 10 µl reactions using the SsoAdvanced™ Universal SYBR® Green and the primers are listed in Table 3.2. Reactions were performed in duplicate and gene expression levels were normalized to the housekeeping gene GAPDH. Relative gene expression was calculated by the comparative CT (threshold cycle) normalized to GAPDH message; ΔCT values were determined by subtracting CT (target) from CT (GAPDH). Expression levels relative to GAPDH were defined as $2^{-\Delta CT}$.

**Table 2.4: Primers used for quantitative PCR characterizing monocytes**

| Primers | Forward 5'-3' | Reverse 5'-3' | Product length |
|---|---|---|---|
| Arg1 | GTG AAG AAC CCA CGG TCT GT | CTG GTT GTC AGG GGA GTG TT | 209bp |
| CD206 | TGC AAA GCT ATA GGT GGA GAGC | ACG GGA GAA CCA TCA CTCC | 164bp |
| Fizz1 | TCC CTC CAC TGT AAC GAA GAC | AGG CAG TTG CAA GTA TCT CCA | 153bp |
| GAPDH | GTG AAG GTC GGT GTG AAC GG | ATG TTA GTG GGG TCT CGC TCC | 245bp |
| IL-10 | TTG AAT TCC CTG GGT GAG AAG | TCC ACT GCC TTG CTC TTA TTT | 95bp |
| IL-6 | AGT TGC CTT CTT GGG ACT GA | TCC ACG ATT TCC CAG AGA AC | 159bp |
| iNOS | GGA ATC TTG GAG CGA GTT GT | CCT CTT GTC TTT GAC CCA GTA G | 99bp |
| TGM2 | CGA ATC CTC TAC GAG AAG TAC AGC | CAG TTT GCG GTT TTG CTT GG | 177bp |

### 2.5.7 Flow cytometric analysis of splenic monocytes and cell sorting

1 million splenocytes were stained with anti-CCR2 antibody for 30 min on ice. After the application of secondary antibody, the cells were washed and blocked with 10% rat serum for 10 min. 1 million cells were stained for other surface markers on ice for 30 min. 20 µl DAPI (100 ng/ml) was added just prior to flow cytometry analysis for excluding dead cells. For intracellular staining, cells were fixed according to the manufacturer's instruction with FOXP3 fix/perm kit. Live/dead fixable Aqua dead cell stain kit was used for dead cell exclusion. Data were acquired using CyAn ADP. Fluorescence minus one control was used for gate strategy to identify interest cells. Neutrophils were identified as $Ly6G^{+}$, DCs were identified as $CD11c^{+}$, and monocytes were gated with the expression of Ly6C and CCR2. Splenic monocytes were sorted by Beckman Coulter MoFlo Astrios cell sorter. $Ly6C^{hi}$ and $Ly6C^{low}$ monocytes or $Ly6C^{+}$ monocytes were sorted by the same gate strategy. The purity of sorted cells were detected and it was > 95%.

**Table 2.5: Antibodies used for splenic monocytes in flow cytometry**

| Host | Mouse immunogen | Fluorochrome | Clone | Supplier |
|---|---|---|---|---|
| Rat | CCR2 | - | MC-21 | M. Mack, Regensburg |
| Mouse | CD11b | PE-Cy7 | M1/70 | eBiosciences |
| Rat | CD11b | PerCP-Cy5.5 | M1/70 | BD Pharmingen |
| Rat | CD11c | PE-Cy7 | HL3 | BD Pharmingen |
| Rat | CD11c | Biotinylated | HL3 | BD Pharmingen |
| Rat | CD80 | FITC | 16-10A1 | BD Pharmingen |
| Rat | CD86 | PE | GL1 | BD Pharmingen |
| Mouse | Gr1 | PerCP-Cy5.5 | RB6-8C5 | BD Pharmingen |
| Rabbit | iNOS | - | Polyclonal | BD Biosciences |
| Rat | Ly6C | FITC | AL-21 | BD Pharmingen |
| Rat | Ly-6G | PE | IA8 | BD Pharmingen |
| Rat | Ly-6G | BV421 | IA8 | Biolegend |
| Mouse | MHC class II | Biotinylated | I-Ab, AF6-120.1 | BD Pharmingen |

**Table 2.6: Secondary labelling for splenic monocytes in flow cytometry**

| **Host** | **Reactivity** | **Fluor chrome** | **Supplier** |
|---|---|---|---|
| Donkey | Rat IgG | AF 647 | Jackson ImmunoResearch Laboratories |
| Goat | Rabbit IgG | Pacific Blue | Molecular probes |
| - | - | Streptavidin conjugated APC Cy7 | BD Pharmingen |

### 2.5.8 Flow cytometric analysis of peritoneal macrophages and cell sorting

One million peritoneal cells were stained with anti-CCR2 antibody for 30 min on ice. After the application of secondary antibody, the cells were washed and blocked with 10% rat serum for 10 min. Cells were stained for surface markers on ice for 30 min. DAPI was added just prior to flow cytometric analysis for exclusion of dead cells. Data were acquired using CyAn ADP. Fluorescence minus one control was used for gate strategy. Neutrophils were identified as $Ly6G^+$, B cells were identified as $CD19^+$, eosinophils were excluded from macrophages by the high side scatter (SSC) as previously described [3]. Peritoneal monocytes and macrophages were gated with the expression of Ly6C and CCR2 and sorted by MoFlo Astrios cell sorter. The sorted cells were collected in 1 ml FACS buffer and spun down at 500 g for 5 min. Cells were lysed in RNA isolation buffer and frozen at -80 °C. The purity of sorted cells was determined and the obtained purity was always > 95%.

**Table 2.7: Primary antibodies used for peritoneal macrophages in flow cytometry**

| Host | Mouse immunogen | Fluorochrome | Clone | Supplier |
|---|---|---|---|---|
| Rat | CCR2 | - | MC21 | M. Mack, Regensburg |
| Mouse | CD11b | PEcy7 | M1/70 | eBioscience |
| Rat | CD19 | APCy7 | ID3 | BD Pharmingen |
| Rat | CD19 | FITC | ID3 | BD Pharmingen |
| Rat | F4/80 | Pecpcy5.5 | BM4 | Biolegend |
| Rat | Ly6C | FITC | AL-21 | BD Pharmingen |
| Mouse | Ly6C | APC | HK1.4 | BD Pharmingen |
| Rat | Ly6G | PE | IA8 | BD Pharmingen |
| Rat | Ly6G | BV421 | IA8 | Biolegend |
| Mouse | MHC class II | biotinylated | I-Ab, AF6-120.1 | BD Pharmingen |

**Table 2.8: Secondary labelling for peritoneal macrophages in flow cytometry**

| Host | Reactivity | Fluorochrome | Supplier |
|---|---|---|---|
| - | - (MHC II) | Streptavidin conjugated APC Cy7 | BD Pharmingen |
| Donkey | Rat (CCR2) | AF 647 | Jackson ImmunoResearch Laboratories |

### 2.5.9 Intracellular staining of peritoneal cavity cells

For intracellular flow cytometry analysis, 1 million cells were stained for cell surface marker staining. Then cells were fixed and permeabilized according to the manufacturer's instruction with FOXP3 fix/perm kit. Live/dead fixable Aqua dead cell stain kit was used for dead cell exclusion. Data were acquired using a CyAn ADP flow cytometer.

**Table 2.9: Antibodies used in intracellular flow cytometry**

| Host | Mouse immunogen | Fluorochrome | Clone | Supplier |
|---|---|---|---|---|
| Sheep | Arg1 | PE | polyclonal | R&D |
| Mouse | CD11b | PEcy7 | M1/70 | eBioscience |
| Armenian Hamster | CD11c | FITC | HL3 | BD Pharmingen |
| Rat | CD19 | APCy7 | ID3 | BD Pharmingen |
| Rat | F4/80 | Pecpcy5.5 | BM4 | Biolegend |
| Rabbit | iNOS | - | polyclonal | BD Biosciences |
| Mouse | Ly6C | APC | HK1.4 | BD Pharmingen |
| Rat | Ly6G | PE | IA8 | BD Pharmingen |
| Rat | Ly6G | BV421 | IA8 | Biolegend |
| Rabbit | Ki67 | - | polyclonal | Abcam |

### 2.5.10 Spleen lysate for ELISA analysis

Spleen cut was weighed and smashed using the lid of Ependorff tube. The spleen lysis buffer was added at volume of 1ml per 1g spleen cut. Cells were lysed for 10 min on ice and cell debris were spin down. Supernatant was collected and froze at -80 °C.

### 2.5.11 IL-1β ELISA

BMDMs were cultured in 96 well plate at concentration of $2x10^5$ and primed with 200 ng/ml LPS for 3 h. Cells were then infected with *L. monocytogenes* at MOI 5, 10, 20, 40 for 45 min. Extracellular *L. monocytogenes* were killed by adding medium with 10 µg/ml Gentamicin. Cell culture supernatant was collected and froze at -80 °C.

96-well ELISA plates were coated with goat anti-mouse-IL-1β monoclonal antibody (diluted 1:400) overnight at 4 °C (coating buffer 50 µl/well). After washing in PBS/0.05% tween three times, the plate was blocked with PBS/1% BSA at room temperature (200 µl/well) for 2 h. The plate was washed in PBS/0.05% tween three times and the samples for IL-1β analysis were added in duplicate and incubated at 4 °C overnight. The plate was washed in PBS/0.05% tween three times, then biotinylated hamster anti-mouse-IL-1β polyclonal antibody (diluted 1:500) was added with 50 µl per well and kept at room temperature for 2 h. The plate was washed in PBS/0.05% tween three times before streptavidin HRP (diluted 1:5000) was added and incubated at room temperature for 30 min. TMB substrate was added to all wells at 50 µl, 3M sulfuric acid was added at 50 µl to stop the reaction. Optical density was read on plate reader at 450 nm. Recombinant IL-1β protein range from 2500 pg/ml to 31 pg/ml was used for standard curve.

### 2.5.12 Western blot

Cell culture supernatant and cell lysates were subjected to NuPAGE™ gel and subsequently transferred to nitrocellulose membranes by iBlot™ 2 dry blotting system. The membranes were shipped to University of Queensland and were immunoblotted with anti-IL-1β, anti-caspase-1, anti-NLRP3 antibodies. The anti-IL-1β antibody (clone P028) was used to detect both pro- and cleaved IL-1β. Pro-IL-1β 37kD and cleaved IL-1β 17kD can be discriminated by protein size.

**Table 2.10: Antibodies for ELISA and Western blot analysis**

| Host | Mouse immunogen | Catalogue number | Clone | Supplier |
|---|---|---|---|---|
| Mouse | Caspase-1 p20(mono) | AF-20B-0042-C100 | P006 | Sapphire Bioscience |
| Goat | IL-1β (polyclonal) | AF-401-NA | P028 | R&D Biosystems |
| Mouse | NLRP3 (Cryo-2) (mono) | AG-20B-0014 | P036 | Adipogen |
| Armenian Hamster | Anti-Mouse/Rat IL-1β Purified | 14-7012-81 | B122 | eBioscience |
| Rabbit | IL-1β biotinylated antibody | 13-7112-81 | polyclonal | eBioscience |
| Mouse | Mouse IL-1β Recombinant Protein | 14-8012-62 | - | eBioscience |

### 2.5.13 *L. monocytogenes* CFSE labelling

*L. monocytogenes* were labelled with 5 mM of the dye carboxyfluorescein diacetate succinimidyl ester (CFSE) for 40 min. After wash with PBS for two times, bacteria were resuspended in PBS and used for macrophage infection.

### 2.5.14 *L. monocytogenes* phagocytosis assay

Macrophages were infected with CFSE labelled *L. monocytogenes* at MOI of 10. After 40 min of infection, cells were washed twice with PBS, the cells were then incubated with medium supplemented with 10 μg/ml gentamicin for 1 h to kill extracellular bacteria. Cells were fixed with acetone and macrophages were labelled with anti-CD11b biotin antibody and Streptavidin Alexa Fluor 546. Cells were washed twice with PBS, and counterstained with DAPI. Coverslips were mounted on slides using fluorescent mounting medium. Pictures were taken with Olympus BX50 microscopy. Percentage of phagocytosis = (bacteria inside macrophages/ total number of bacteria counted) x100.

### 2.5.15 *L. monocytogenes* phagosomal escape assay

Following *L. monocytogenes* infection, cells were harvested after incubation for 3 h. Cell were washed, fixed with ice-cold acetone, and washed with PBS. Then cells were permeabilized with 0.25% Triton X-100 for 10 min at room temperature. After permeabilization, cells were washed and blocked in blocking buffer for 20 min. Actin filaments were labelled with phalloidin conjugated Alexa Fluor 594 antibody and counterstained with DAPI. The cells were washed three times in PBS and mounted using fluorescent mounting medium. Confocal microscopy was performed using spinning disk and z-stacks were taken in slices of 1 μm. Twenty macrophages per slide were examined for the presence of bacteria co-localizing with phalloidin. Phagosome escape was expressed as the percentage of phalloidin positive bacteria.

**Tabel 2.11: Antibodies used in immunofluorescence**

| Host | Reactivity | Fluor chrome | Supplier |
|---|---|---|---|
| Rabbit | mouse | Anti-LAMP1 antibody (1D4B) | BD Pharmingen |
| - | - | Phalloidin conjugated Alexa Fluor 594 | Thermo Fisher |

**Tabel 2.12: Secondary antibodies used in immunofluorescence**

| Host | Reactivity | Fluor chrome | Supplier |
|---|---|---|---|
| Goat | rabbit | Alexa Fluor 594 | Molecular probes |
| - | - | Streptavidin Alexa Fluor 546 | BD Pharmingen |

### 2.5.16 DFTD cells phagocytosis assay

BMDMs (effector cells) were labelled with CellTrace™ Violet (CTV). DFTD cells (target cells) were labelled with 5(6)-Carboxyfluorescein diacetate N-succinimidyl ester (CFSE) according to the manufacturer's instruction. BMDMs were suspended in cell culture medium at $10^6$/ml. A 100 µl doubling dilution of BMDMs was performed to obtain effector to target ratios of 10:1, 5:1 with $10^4$ target DFTD cells. The phagocytosis assays were performed using duplicates or triplicates and incubated for 4 and 24 h at 37 °C and for 4 h at 4 °C for the negative control. The plates were analysed on a BD Canto II flow cytometer. To calculate phagocytosis, three regions were identified. R1, total $CFSE^+$ tumour cells = total tumour cells. R2, $CFSE^+/CTV^+$ cells = tumour cells phagocytosed by macrophages. R3, $CFSE^+/CTV^-$ cells = free tumour cells. The formula used for calculating phagocytosis was: percentage of phagocytosis= (R2/R1) x 100%.

For the 24 h incubation, in order to avoid complication of tumour cell division or been phagocytosed by macrophages, bead assay was used in enumerating tumour cells and macrophages in each treatment. For the bead count method, 5,000 beads were added in wells before the analysis of flow cytometry. The number of cells and beads were recorded together and the number of cells were calculated according to the ratio of collected beads to 5,000. The number of DFTD cells remaining = (5000/number of beads collected) x R3 cells. Flow cytometry was conducted at least three times in duplicate or triplicate wells.

### 2.5.17 Cell staining and confocal microscopy

CTV labelled BMDMs were grown in 24 well plates on 0.2% gelatine coated 12 mm coverslips. CFSE labelled DFTD cells were added when the coverslip was almost confluent with BMDMs. After 4 and 24 h incubation, cells were gently washed with PBS. Cells were permeabilized with 0.25% Triton X-100 for 20 min and blocked with a 1% BSA for 20 min. Antibody staining was conducted for 30 min with a rabbit anti-mouse-LAMP1 antibody. The goat anti rabbit

Alexa Fluor 594 secondary antibody was applied for 30 min. The cells were washed three times in PBS and mounted using Dako mounting medium. Confocal microscopy was performed using spinning disk and z-stacks were taken in slices of 1 µm.

### 2.5.18 Griess assay for detection of nitrite

The contents of nitrate and nitrite in the cell culture media were determined by the Griess assay as described previously [223]. 50 µl cell culture medium was mixed with 50 µl 1% sulphanilamide in 2.5% $H_3PO_4$, then 50 µl Griess reagent (0.1% napthylenediamide dihydrochloride) and incubated at room temperature for 10 min. The absorbance at 540 nm was measured by a microplate reader and compared to a standard nitrite curve using sodium nitrite.

### 2.5.19 Software/ Programmes

Data analysis of flow cytometry was performed using FlowJo V10.1.
Immunofluorescence assay was analysed by Image J.
Statistical analyses were performed in Graphpad Prism5.

### 2.5.20 Statistical analysis

Statistical significance was determined by Mann-Whitney test in Chapter 3 and 4. Student's unpaired t-test was used in Chapter 5. Each experimental group was compared to B6.WT controls. P values of less than 0.05 were considered statistically significant.

# Chapter 3

## Lack of TNF leads to alternative activation in splenic macrophages during *L. monocytogenes* infection

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### 3.1 Introduction

TNFα (TNF) is a cytokine that plays an important role in inflammatory responses. TNF is mainly produced by macrophages and T cells during infection [5]. Macrophages are plastic in their differentiation as they can become either classically activated macrophages (CAMs) or alternatively activated macrophages (AAMs) in response to the environment in the tissues [41]. As a pro-inflammatory cytokine, it has been documented that TNF inhibits AAMs differentiation in both *Leishmania major* infections [35] and tumour models [34]. The role of TNF in macrophage differentiation during bacterial infection such as *L. monocytogenes* is unknown.

The spleen is the major target tissue of systemic *L. monocytogenes* infection. $Ly6C^{hi}CD11b^{+}$ monocytes are recruited into spleen and differentiate into macrophages or dendritic cells during *L. monocytogenes* infection [69]. CCR2 is required for recruitment of monocytes, thus $CCR2^{-/-}$ mice are susceptible to *L. monocytogenes* infection due to the lack of $Ly6C^{hi}CD11b^{+}$ monocyte infiltration to spleen [69]. The recruited $Ly6C^{hi}CD11b^{+}$ monocytes produce high levels of NO and TNF, which are required to eliminate the bacterial infection [69]. Neutrophils are essential during the early control phase of *L. monocytogenes* infection via phagocytosis [224]. Neutrophils release reactive nitrogen and oxygen intermediates to kill *L. monocytogenes* directly and modulate tissue damage with overproduction of these mediators [224]. It has been shown that the depletion of neutrophils using an anti-Gr-1 (RB6-8C5) monoclonal antibody results in a severe bacterial burden following infection with *L. monocytogenes* [225].

We hypothesised that TNF inhibits splenic macrophages differentiation into AAMs during infection *with L. monocytogenes*. We compared the innate immune responses in spleen, including monocyte differentiation, neutrophil composition, cytokine IL-1β release, between TNF deficient mice and wild-type controls with *L. monocytogenes* infection. Our study showed that splenic macrophages from TNF deficient mice were biased toward AAM phenotype following *L. monocytogenes* infection, demonstrating the key role of TNF and CAMs in control of intracellular bacterial infection. Moreover, splenic neutrophil numbers and IL-1β production were enhanced in response to the impaired bacterial clearance in TNF deficient mice.

## 3.2 Results

### 3.2.1 B6.TNF$^{-/-}$ mice are susceptible to *L. monocytogenes* infection.

The bacterial burden in spleen and liver was examined at the indicated days post infection to investigate the role of TNF in anti- *L. monocytogenes* responses. As shown in Figure 3.2.1A, 3.2.1B, bacterial loads in B6.WT and B6.TNF$^{-/-}$ mice were similar in spleen and liver at day 1 post infection. It suggested that these two strains had the similar initiation of infection. Compared with WT controls, B6.TNF$^{-/-}$ mice displayed increased bacterial burden in spleen but not in liver at day 2 of infection (Fig 3.2.1A, 3.2.1B). B6.TNF$^{-/-}$ mice showed aggressive infection in spleen and liver at 3 days and 4 days after infection (Fig 3.2.1A, 3.2.1B), suggesting the high susceptibility in B6.TNF$^{-/-}$ mice with *L. monocytogenes* infection.

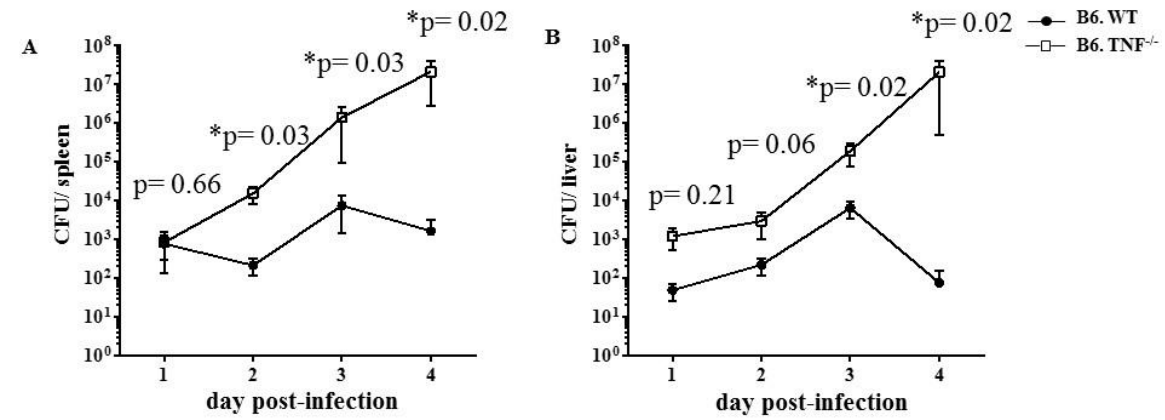


**Figure 3.2.1: B6.TNF$^{-/-}$ mice had increased bacterial loads in spleen and liver after infection with *L. monocytogenes* intraperitoneally.** B6.WT and B6.TNF$^{-/-}$ mice were i.p injected with *L. monocytogenes*. At the indicated days after infection, CFUs were assayed by culturing organ homogenates on brain heart infusion (BHI) plates overnight. Bacterial loads in spleen (A) and liver (B) from B6.WT and B6.TNF$^{-/-}$ mice infected with *L. monocytogenes* at indicated days. n= 4-6 mice from two independent experiments, error bars indicate SEM, * $p < 0.05$.

### 3.2.2. Splenic neutrophil, DC, monocyte subpopulation kinetics in B6.WT and B6.TNF$^{-/-}$ mice after *L. monocytogenes* infection.

To understand the protective effects of TNF in response to *L. monocytogenes* infection, we analysed the composition of innate immune cells, including neutrophils, DCs and monocytes. Under steady-state conditions (e.g. day 0), total numbers of neutrophil and DC were comparable in B6.WT and B6.TNF$^{-/-}$ mice (Fig 3.2.2A, 3.2.2B), suggesting that the development of neutrophils and dendritic cells were not affected by TNF deficiency. Upon *L.*

*monocytogenes* infection at day 1 and day 2, no significant differences in the numbers of neutrophil and DC were identified. At day 3 and day 4 post infection, the total numbers of neutrophil in B6.TNF$^{-/-}$ mice were higher than WT controls (Fig 3.2.2A), but no difference was identified in the number of DCs (Fig 3.2.2B). It indicated that TNF signalling is involved in neutrophil accumulation but not DCs kinetics in response to *L. monocytogenes* infection.

Monocytes were identified by the expression of Ly6C antigen. As shown in Figure 2C, CCR2$^{+}$Ly6C$^{+}$ monocytes were grouped into Ly6C$^{hi}$R1 and Ly6C$^{low}$R2 monocytes according to the expression of Ly6C. The numbers of CCR2$^{+}$Ly6C$^{+}$ monocytes were similar in B6.WT and B6.TNF$^{-/-}$ mice (Fig 3.2.2D), suggesting TNF does not affect the recruitment of CCR2$^{+}$Ly6C$^{+}$ monocytes. Ly6C$^{hi}$R1 monocytes are the most abundant subset of CCR2$^{+}$Ly6C$^{+}$ monocytes. The numbers of Ly6C$^{hi}$R1 monocytes were similar between two strains of mice (Fig 3.2.2E). The numbers of Ly6C$^{low}$R2 monocyte were reduced in B6.TNF$^{-/-}$ mice after day 3 and day 4 infection, compared with WT controls (Fig 3.2.2F). It indicated that TNF was involved in balancing the monocyte subsets in response to *L. monocytogenes* infection.

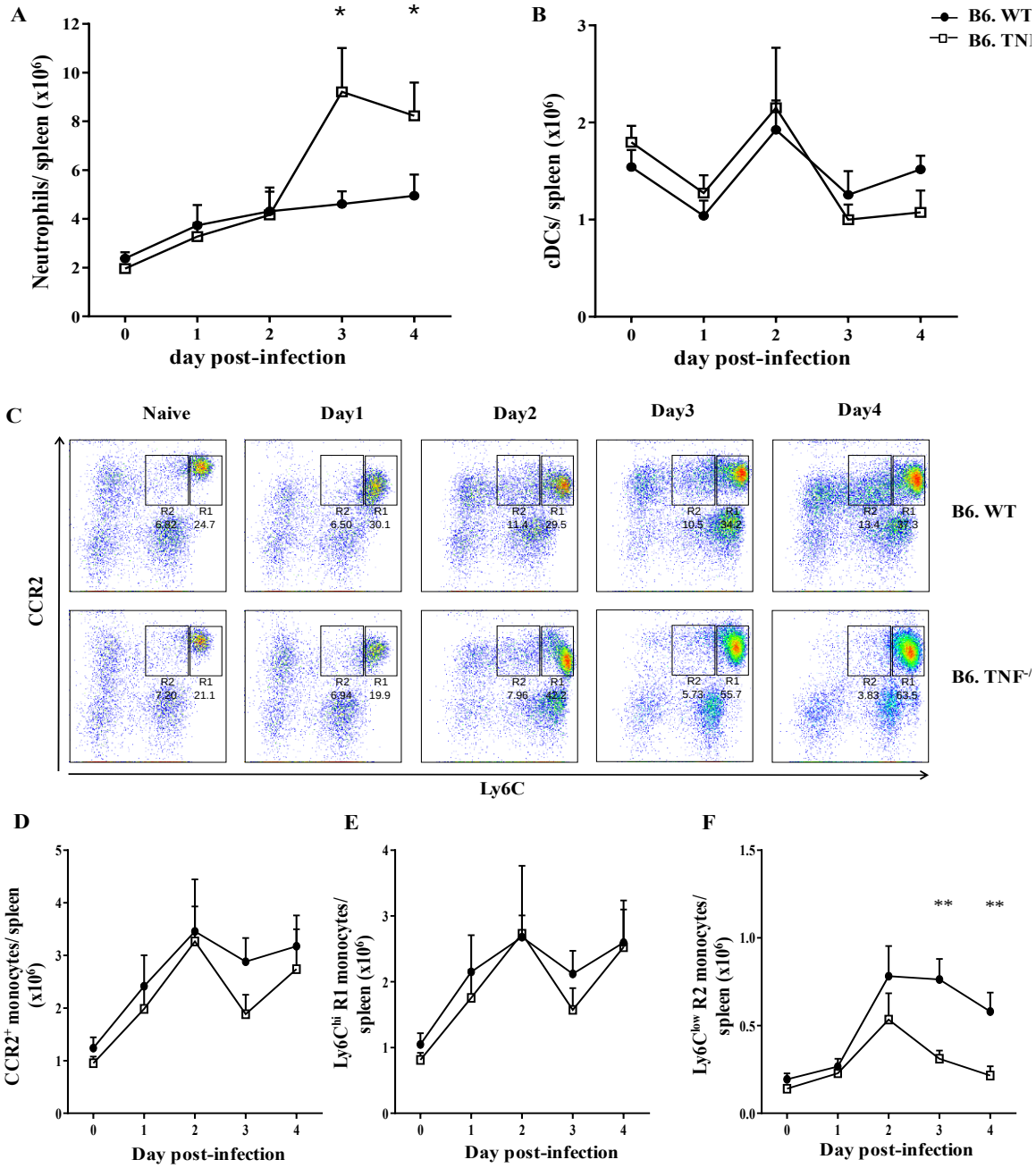


**Figure 3.2.2: Kinetics of neutrophil, DC and monocyte subsets following *L. monocytogenes* infection.** The analysis of splenic cell subsets from B6.WT and B6.TNF$^{-/-}$ mice at indicated day post infection with 1,000 *L. monocytogenes*. Total numbers of splenic neutrophil, DC and monocyte were analysed by flow cytometry. The numbers of neutrophil (A) and DC (B) following *L. monocytogenes* infection were quantified. (C) Representative flow plots of CCR2$^{+}$Ly6C$^{+}$ monocytes from B6.WT and B6.TNF$^{-/-}$ mice. CCR2$^{+}$Ly6C$^{+}$ monocytes were gated into Ly6C$^{hi}$R1 and Ly6C$^{low}$R2 monocytes. Quantification of total numbers of CCR2$^{+}$ monocyte (D), Ly6C$^{hi}$R1 (E) and Ly6C$^{low}$R2 (F) monocyte from uninfected or *L. monocytogenes* infected B6.WT and B6.TNF$^{-/-}$ mice. Each time point represents 6-12 mice from two of three independent experiments (error bars indicate SEM), * $p< 0.05$, ** $p< 0.01$.

### 3.2.3. TNF deficiency does not alter iNOS expression in Ly6C$^{hi}$R1 and Ly6C$^{low}$R2 monocytes.

As demonstrated before [69], iNOS is the important effector molecule produced by Ly6C$^{hi}$ inflammatory monocytes with *L. monocytogenes* infection. We next investigated the role of TNF in iNOS expression in Ly6C$^{hi}$R1 and Ly6C$^{low}$R2 monocytes during *L. monocytogenes* infection. The expression of iNOS was examined by intracellular flow cytometry. Ly6C$^{hi}$R1 monocytes in B6.TNF$^{-/-}$ mice expressed similar level of iNOS with B6.WT mice (Fig 3.2.3), indicating the intact capacity in iNOS production in the deficient of TNF. In the comparison of iNOS expression in Ly6C$^{hi}$R1 and Ly6C$^{low}$R2 monocytes within the same genotype of mice, Ly6C$^{hi}$R1 monocytes expressed increased amount of iNOS compared to Ly6C$^{low}$R2 monocytes in both two genotypes. It indicated that Ly6C$^{hi}$R1 monocytes were the main producers in iNOS synthesis and iNOS expression was independent of TNF following *L. monocytogenes* infection.

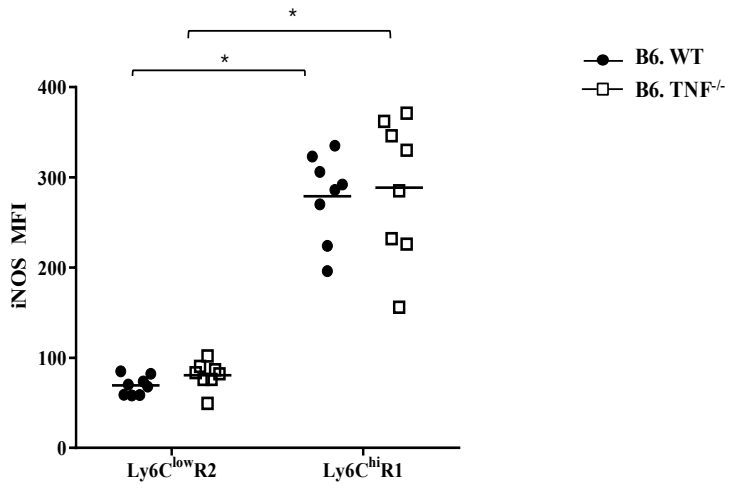


**Figure 3.2.3: iNOS expression in Ly6C$^{hi}$R1 and Ly6C$^{low}$R2 monocytes after day 3 infection.** B6.WT and B6.TNF$^{-/-}$ mice infected with *L. monocytogenes* for 3 days, the expression of iNOS in iNOS from Ly6C$^{hi}$ R1 and Ly6C$^{low}$ R2 monocytes were detected by intracellular flow cytometry. MFI of iNOS in Ly6C$^{hi}$ R1 and Ly6C$^{low}$R2 monocytes in B6.WT and B6.TNF$^{-/-}$ mice. n=8 mice from two independent experiments, * $p < 0.05$.

### 3.2.4. AAM differentiation from B6.TNF$^{-/-}$ mice following *L. monocytogenes* infection.

Our results showed that iNOS synthesis is independent of TNF during *L. monocytogenes* infection. We then compared CAM and AAM differentiation using qPCR profiling of subset specific gene expression. The Ly6C$^{hi}$R1 monocyte and Ly6C$^{low}$R2 monocytes from B6.WT

and B6.TNF$^{-/-}$ mice were sorted at 3 days post infection with *L. monocytogenes*. The expression of CAM markers iNOS, IL-6 and AAM markers Arg1, CD206, TGM2, Fizz1, IL-10 were detected by qPCR. The iNOS expressions in Ly6C$^{hi}$R1 and Ly6C$^{low}$R2 monocytes were similar between B6.WT and B6.TNF$^{-/-}$ group, which is consistent with the flow cytometry results (Fig 3.2.3). Compared with WT controls, the expression of Arg1, TGM2 in Ly6C$^{hi}$R1 monocytes were increased in B6.TNF$^{-/-}$ mice (Fig 3.2.4B, 3.2.4E). Whereas the expression of mannose receptor CD206 was decreased in Ly6C$^{hi}$R1 monocytes in B6.TNF$^{-/-}$ mice (Fig 3.2.4D). These results indicated that TNF deficiency altered AAM differentiation of Ly6C$^{hi}$R1 monocytes in response to *L. monocytogenes* infection.

For the analysis of gene expression between Ly6C$^{hi}$R1 monocytes and Ly6C$^{low}$R2 monocytes within the same genotype of mice, the expression of AAM markers (CD206, TGM2, Fizz1, IL-10) were upregulated in Ly6C$^{low}$R2 monocytes (Fig 3.2.4D-G). It suggested that Ly6C$^{low}$R2 monocytes were AAMs, which were required for tissue modelling and repair from infection associated pathology. Ly6C$^{hi}$R1 monocytes from B6.TNF$^{-/-}$ mice displayed AAM bias than wild-type controls.

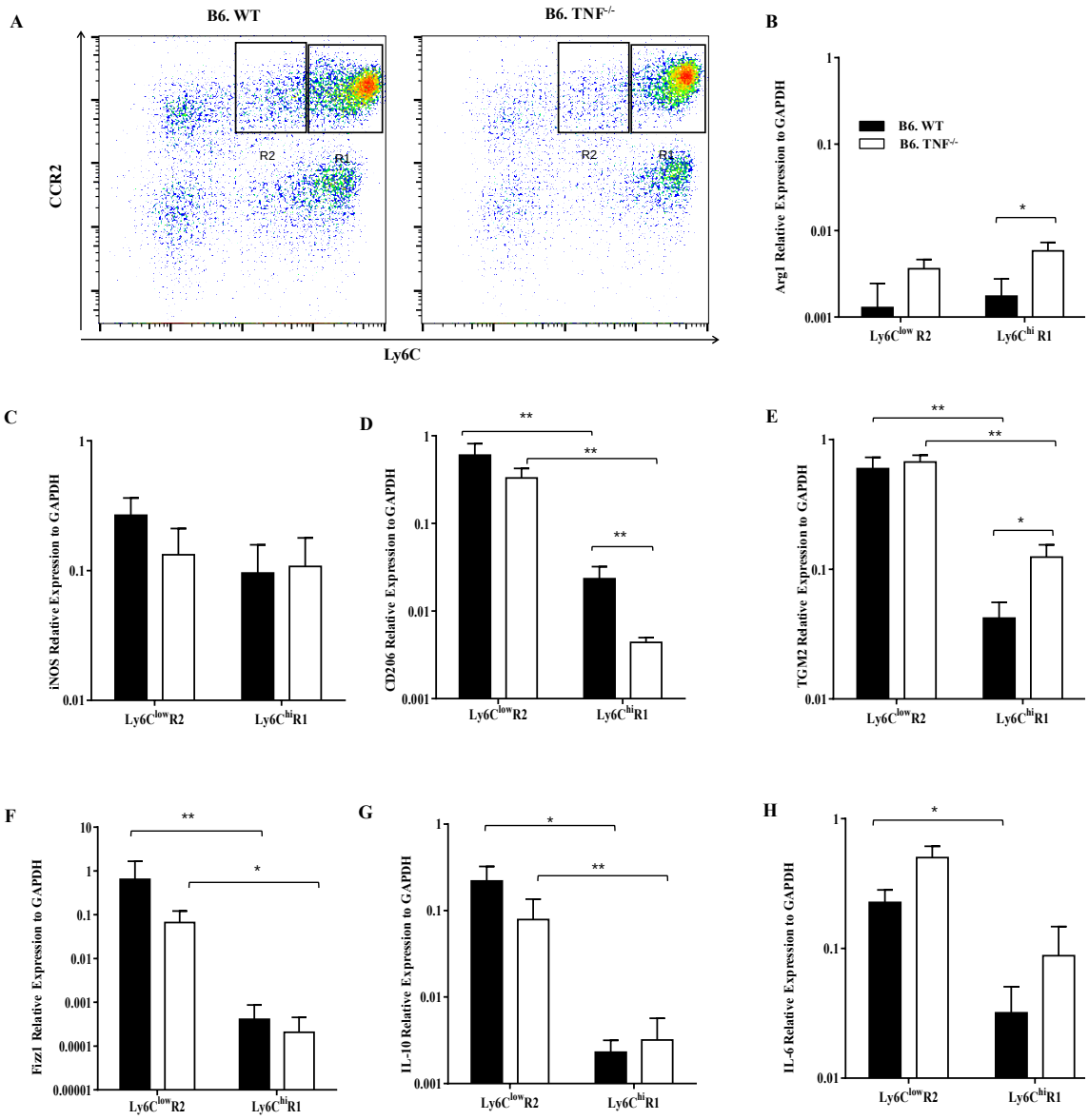


**Figure 3.2.4: Expression of activation markers by Ly6C$^{hi}$R1 and Ly6C$^{low}$R2 monocytes in the spleen at 3 days post infection.** Ly6C$^{hi}$ R1 and Ly6C$^{low}$ R2 monocytes were sorted from spleen of B6.WT and B6.TNF$^{-/-}$ mice infected with *L. monocytogenes* for 3 days. Gene expression of Ly6C$^{hi}$R1 and Ly6C$^{low}$R2 monocytes was analysed by qPCR. Representative plots of R1 and R2 monocytes for cell sorting (A). The relative mRNA expression of Arg1, iNOS, CD206, TGM2, Fizz1, IL-10, IL-6 (B-H) in Ly6C$^{hi}$R1 and Ly6C$^{low}$R2 monocytes. n= 3-8 mice for each group, error bars were SEM, * $p < 0.05$, ** $p < 0.01$.

### 3.2.5. TNF deficiency in Ly6C$^{+}$ monocytes impairs anti- *L. monocytogenes* response.

Our result showed the increased expression of Arg1 in Ly6C$^{hi}$R1 monocytes in the deficiency of TNF. Due to the low yield of rare cells (Ly6C$^{hi}$ and Ly6C$^{low}$ monocytes), we isolated Ly6C$^{+}$ ( combined Ly6C$^{hi}$ and Ly6C$^{low}$ monocytes) monocytes. We then investigated the bacterial load, Arg1 and iNOS expression in Ly6C$^{+}$ monocytes. The bacterial loads in TNF deficient Ly6C$^{+}$ monocytes were increased than WT controls (Fig 3.2.5A), suggesting the impairment of *L. monocytogenes* elimination of Ly6C$^{+}$ monocytes in the deficiency of TNF. The expressions of Arg1 and iNOS in Ly6C$^{+}$ monocytes from TNF deficient mice were enhanced, though did not reach significant difference (Fig 3.2.5B, 3.2.5C).

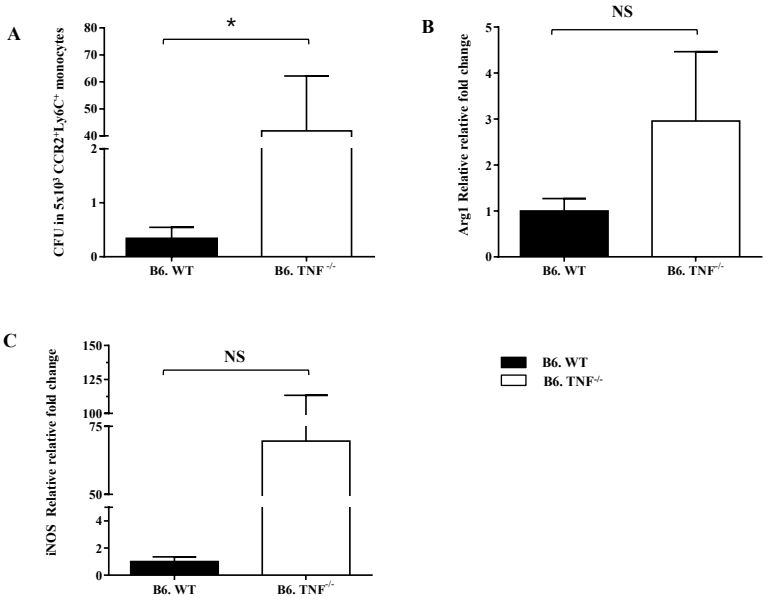


**Figure 3.2.5: Bacterial loads and expression of Arg1 and iNOS in Ly6C$^{+}$ monocytes at 3 days post infection.** Ly6C$^{+}$ monocytes were sorted from spleen of B6.WT and B6.TNF$^{-/-}$ mice infected with *L. monocytogenes* for 3 days. Bacterial load in Ly6C$^{+}$ monocytes from B6.WT and B6.TNF$^{-/-}$ mice after BHI agar plates culturing for 24 hours (A). The relative mRNA expression of Arg1 (B) and iNOS (C) in Ly6C$^{+}$ monocytes was determined and normalized to the housekeeping gene GAPDH. n = 3-5 mice/ genotype; * $p < 0.05$, NS not significant difference.

### 3.2.6. TNF deficiency leads to reduced neutrophil composition in the bone marrow during *L. monocytogenes* infection.

Neutrophils are generated and matured in the bone marrow, then rapidly recruited into tissues in response to inflammatory stimulus [226]. Increased neutrophil numbers were observed in B6.TNF$^{-/-}$ spleen during *L. monocytogenes* infection (Fig 3.2.2A), we next examined whether it is due to the increased recruitment form bone marrow in B6.TNF$^{-/-}$ mice. Naïve B6.WT, B6.TNF$^{-/-}$ mice had similar total number and percentage of neutrophils in the bone marrow

(Fig 3.2.6A, 3.2.6B). Following *L. monocytogenes* infection for 3 and 4 days, B6.TNF$^{-/-}$ mice had both reduced number and percentage of neutrophils compared to B6.WT littermates (Fig 3.2.6A, 3.2.6B). The reduced neutrophil numbers in the bone marrow were consistent with the increased neutrophil numbers in the spleen from B6.TNF$^{-/-}$ mice following *L. monocytogenes* infection.

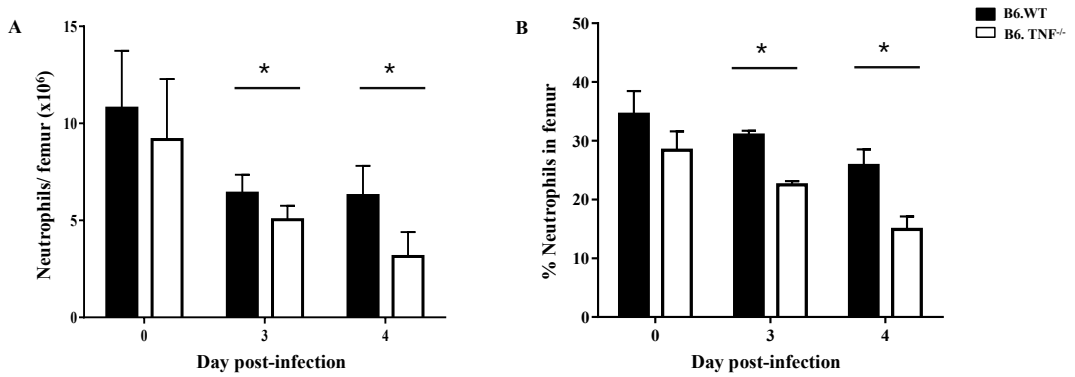


**Figure 3.2.6: TNF deficiency results in reduced neutrophils composition in the bone marrow after *L. monocytogenes* infection.** The bone marrow cells were harvested from femurs of B6.WT, B6.TNF$^{-/-}$ mice under naïve or *L. monocytogenes* infected conditions. Total number of neutrophils in the bone marrow (A) and frequency of neutrophils in the bone marrow (B) were analysed by flow cytometry. n= 6-8 mice from two independent experiments, error bars indicate SEM, * $p < 0.05$.

### 3.2.7. TNF deficiency results in enhanced IL-1β release during *L. monocytogenes* infection *in vivo*.

The increased neutrophils in spleen in B6.TNF$^{-/-}$ mice suggested an enhanced inflammatory response to *L. monocytogenes* infection compared to WT controls. To investigate further, expression of the inflammatory cytokine IL-1β was determined following *L. monocytogenes* infection. In order to investigate the role of TNF in IL-1β release during *L. monocytogenes* infection, we measured IL-1β production in spleen lysate (method referred to section 2.4.21) and peritoneal fluids from *L. monocytogenes* infected B6.WT and B6.TNF$^{-/-}$ mice at indicated days. IL-1β production in spleen lysate from B6.TNF$^{-/-}$ mice was increased at day 3 infection (Fig 3.2.7A). Peritoneal lavage from B6.TNF$^{-/-}$ mice had increased IL-1β level at day 2 and day 3 of infection compared to WT controls (Fig 3.2.7B).

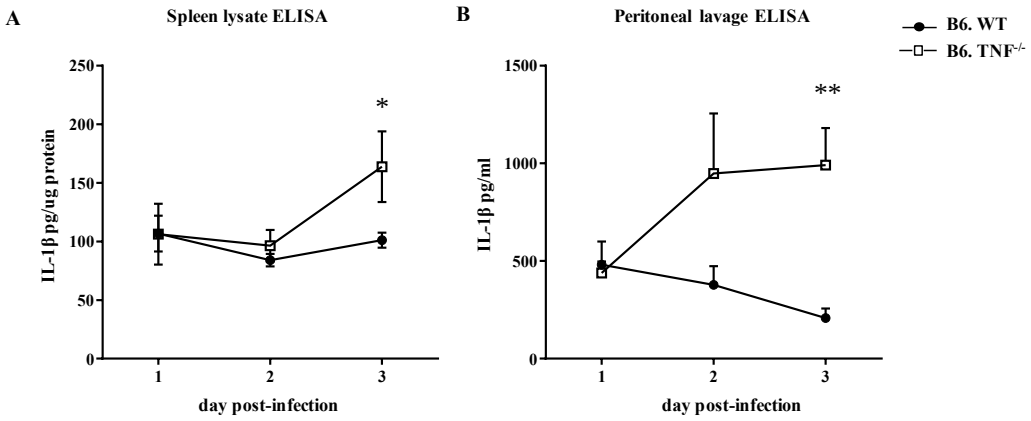


**Figure 3.2.7: Deficient of TNF results in enhanced IL-1β production in response to *L. monocytogenes* infection *in vivo*.** B6.WT and B6.TNF$^{-/-}$ mice were infected with *L. monocytogenes* for indicated days, spleen lysate and peritoneal fluids were collected at indicated days. The concentration of IL-1β in spleen lysate (A) and peritoneal fluids (B) was measured using ELISA. n= 6-10 mice/genotype, error bars are SEM, * $p < 0.05$, ** $p < 0.01$.

### 3.2.8. TNF deficiency results in enhanced pro-IL-1β production during *L. monocytogenes* infection *in vitro*.

In order to confirm the results *in vivo*, we next examined the role of TNF in IL-1β releasing during *L. monocytogenes* infection *in vitro.* IL-1β production is regulated by inflammasomes which are multiple protein platforms termed NLRP3, ASC, and pro-caspase-1 [138]. The components of inflammasome such as NLRP3, caspase-1, pro-caspase-1 were examined to investigate the role of TNF in IL-1β release. BMDMs from B6.WT and B6.TNF$^{-/-}$ mice were primed with LPS for 3 h and infected with *L. monocytogenes* at MOI 5, 10, 20, 40. The IL-1β concentrations in cell culture supernatants were measured with ELISA. Following *L. monocytogenes* infection at MOI 10, MOI 20 and MOI 40 (Fig 3.2.8A), macrophages from B6.TNF$^{-/-}$ mice released enhanced levels of IL-1β compared to WT controls. These results were consistent with the increased production of IL-1β in B6.TNF$^{-/-}$ mice with *L. monocytogenes* infection *in vivo*.

We next performed western blot of cell culture supernatants and cell lysates to further examine the IL-1β release. Due to some unexpected problems, the western blot was only performed once and further experimentation would be required. The level of IL-1β in cell culture supernatants from B6.TNF$^{-/-}$ BMDMs was higher after *L. monocytogenes* infection (Fig 3.2.8B), which is cautiously consistent with the ELISA results. In the cell lysates, increased levels of pro-IL-1β from TNF deficient BMDMs were identified (Fig 3.2.8B), which is

cautiously consistent with the increased IL-1β levels during *L. monocytogenes* infection. The levels of inflammasome components such as NLRP3, caspase-1, pro-caspase-1 were similar in BMDMs from B6.WT and B6.TNF$^{-/-}$ mice, may indicate that TNF is independent in NLRP3 inflammasome activation during *L. monocytogenes* infection.

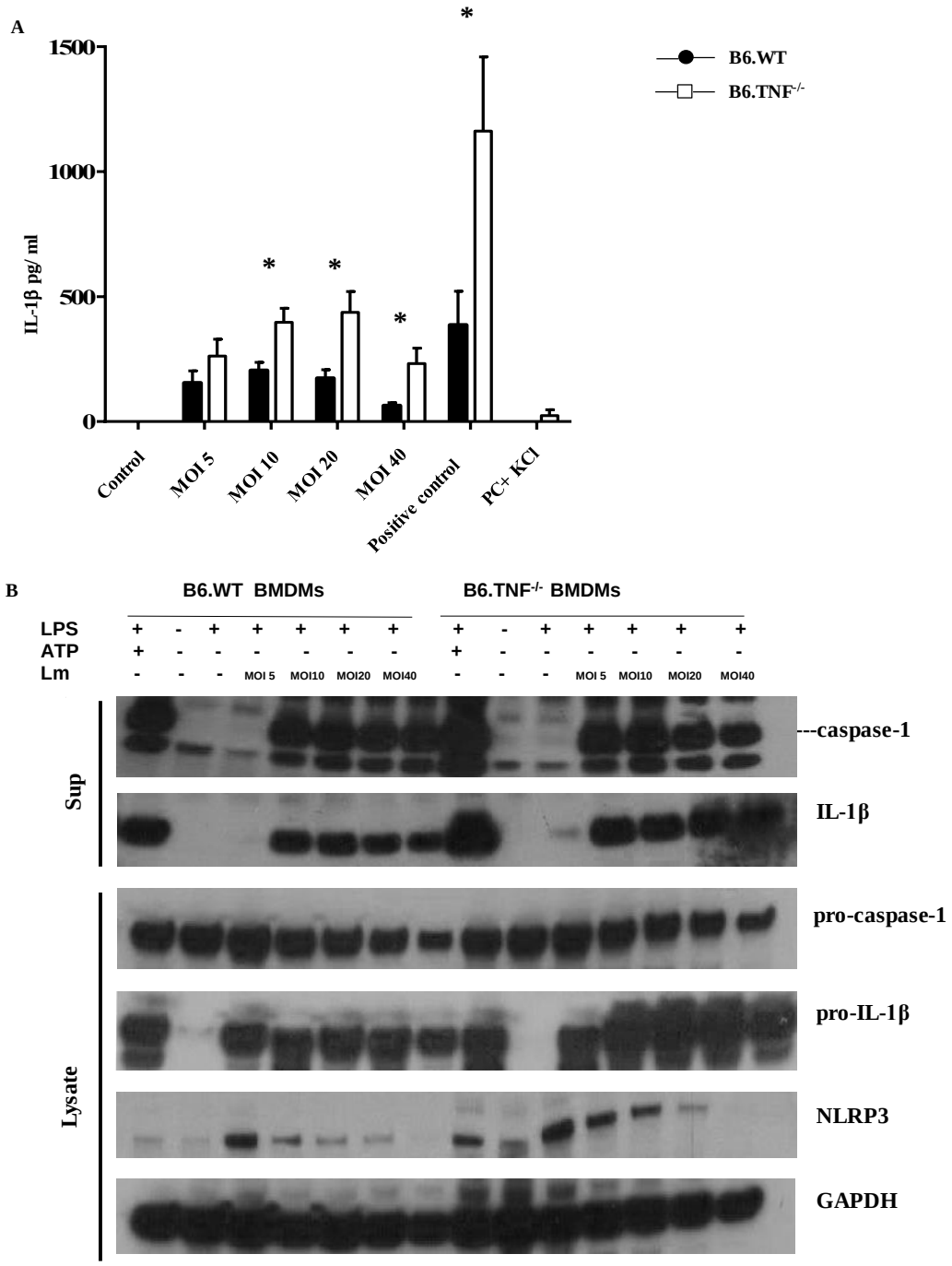


**Figure 3.2.8: TNF absence results in enhanced IL-1β production during *L. monocytogenes* infection *in vitro*.** BMDMs from B6.WT and B6.TNF$^{-/-}$ mice were primed with LPS for 3 h, then infected with *L. monocytogenes* at MOI 5, 10, 20, 40. After washing and incubation in the cell culture

medium with 10ug/ml Gentamycin for 6 hours, the concentrations of IL-1β in cell culture supernatants were examined by ELISA (A). The expressions of pro-caspase-1, pro- IL-1β, NLRP3 and GAPDH in cell lysates and caspase-1, IL-1β in cell culture supernatants were examined by western blot (B). n= 4 from two independent experiments, error bars were SEM, * $p < 0.05$. Note: figure B came from the antibody staining from Kate Schroder's group.

### 3.3 Discussion

The importance of TNF in the elimination of microbial infection has been evidenced in the study of TNFR1 deficient mice during *L. monocytogenes* infection [31]. This chapter showed that TNF deficiency in splenic Ly6C$^{hi}$ monocytes results in AAM bias, which leads to the impaired killing ability of *L. monocytogenes*. It is consistent with the role of TNF in AAM differentiation during parasitic infections [35] and in tumour models [34]. This indicates the essential role of TNF in protection from infection by regulation of macrophage function.

Ly6C$^{hi}$ monocytes that produce large amounts of iNOS and TNF have been shown to be essential in defence against *L. monocytogenes* infection [69]. In combination with IFNγ, TNF promotes macrophage differentiate into classically activated macrophages (CAMs) [111]. In addition, parasite and tumour models have indicated that TNF is a negative regulator of AAM differentiation. TNF deficiency leads to highly expressed Arg1 in macrophages [34, 35]. Results in this chapter indicate that Arg1 was highly expressed in Ly6C$^{hi}$ monocytes of B6.TNF$^{-/-}$ mice following the infection of *L. monocytogenes* (Fig 3.2.6B). This is consistent with the role of TNF in dampening Arg1 expression in parasitic infection and tumour models [34, 35]. TNF inhibits Arg1 expression by suppressing histone acetylation at the Arg1 promoter and enhancer elements [35]. The hyper expressed Arg1 impairs NO release, which leads to parasite proliferation following *L. major* infection [35]. We found that TNF deficiency did not alter the expression of iNOS (Fig 3.2.5, 3.2.6C), but showed impaired *L. monocytogenes* elimination in monocytes (Fig 3.2.6C). The relationship between high expression of Arg1 and reduced NO production needs to be investigated further. Moreover, the mechanism of TNF inhibition of Arg1 expression in macrophages during *L. monocytogenes* infection needs to be investigated. In addition to the enhanced expression of Arg1 in the deficiency of TNF, TGM2 expression is also increased. It is possible that TNF downregulates transcriptional level of Arg1 and TGM2 during *L. monocytogenes* infection. Thus protein levels of Arg1 and TGM2 in TNF deficient splenic macrophages are needed to be investigated in the future. The other AAM markers Fizz1 and IL-10 were unchanged and CD206 was decreased.

In the deficiency of TNF, these Ly6C$^{hi}$ monocytes may not 'typical AAMs' which have high expression of AAM markers. The changes in expression of Arg1, TGM2 and CD206 might lead to functional changes in Ly6C$^{hi}$ monocytes in response to infection of *L. monocytogenes* (see more in discussion of chapter 4).

Ly6C$^{low}$ monocytes are termed resident monocytes and patrol along vascular endothelium and surrounding tissues to combat infection [43, 68]. These monocytes are characterized by high expression of chemokine receptor CX3CR1 and lack expression of CCR2 [68]. Ly6C$^{low}$ monocytes are proposed to be AAMs involved in wound healing at late stage of ischemic myocardium, by expressing vascular-endothelial growth factor (VEGF) [227]. It has been suggested that Ly6C$^{low}$ monocytes initiate a transcriptional program of AAMs in the early stage of *L. monocytogenes* infection, which is consistent with tissue repair [71]. Our results showed the AAM phenotypes of Ly6C$^{low}$ monocytes with high expression of AAM markers (TGM2, CD206, Fizz1 and IL-10) (Fig 3.2.6D-G), which is in line with other models. However, the Ly6C$^{low}$ monocytes in our study expressed CCR2 (Fig 3.2.2C), which has not been shown in other studies. The CCR2 expression suggests that these Ly6C$^{low}$ monocytes are derived from the bone marrow. It has been suggested that Ly6C$^{hi}$ monocytes become Ly6C$^{low}$ monocytes by down regulating Ly6C expression in the blood and bone marrow [228]. In the sterile wound healing model, Ly6C$^{hi}$ monocytes mature into Ly6C$^{low}$ monocytes as 'repair' macrophages [229]. Thus, it appeared that Ly6C$^{low}$ monocytes were generated from Ly6C$^{hi}$ monocytes and that TNF was required when Ly6C$^{hi}$ monocytes matured into Ly6C$^{low}$ monocytes. We found an increased bacterial load in TNF deficient Ly6C$^{+}$ monocytes (Fig 3.2.6A), which might correlate to a reduced transition from Ly6C$^{hi}$ to Ly6C$^{low}$ monocytes. It is not certain whether it is the TNF or the uncontrolled bacterial infection that promotes Ly6C$^{hi}$ monocyte maturation into Ly6C$^{low}$ monocytes.

It has been suggested that TNF induces the expression of CXCL1 (KC) and CXCL2 (MIP-2) [230], which facilitates the homing of neutrophils into infected tissues. The chemokines CXCL1 (KC) and CXCL2 (MIP-2) are required for neutrophil infiltration into infected tissue [231]. The high expression of CXCL1 was reported in the liver from TNF deficient mice with *L. monocytogenes* infection, suggesting CXCL1 may be the chemokine responsible for infiltration of neutrophils in TNF deficiency [232]. The enhanced infiltration of neutrophils in TNF deficient mice in our study might result from the chemokines such as CXCL1 and CXCL2. Neutrophils kill *L. monocytogenes* by phagocytosis and ROS production [233]. The production

of ROS in neutrophil such as $O_2^-$ and $H_2O_2$ are regulated by the formylpeptide peptides binding to the receptor (FPR1) and calcium entry [234]. The overproduction of ROS in neutrophils can also cause tissue damage, as inhibition of ROS production from phagocytes prevents host tissue damage and it is necessary for the resolution of an inflammatory response [235]. The neutrophils lead to tissue damage following *L. monocytogenes* by killing endothelial cells in liver [236]. Thus, the aggressive infiltration of neutrophil in B6.TNF$^{-/-}$ mice might lead to tissue damage, and finally cause high susceptibility during *L. monocytogenes* infection.

Our results showed an increased IL-1β production in B6.TNF$^{-/-}$ mice following *L. monocytogenes* infection, which contradicts previous reports [237].The secretion of IL-1β requires two steps: the first is the synthesis of pro-IL-1β in the cells; the second is activation of caspase-1, which cleaves pro-IL-1β into activated IL-1β [142]. It has been indicated that exogenous TNF induces IL-1β release through NF-κB activation, as the drugs that inhibit NF-κB activation result in impaired pro-IL-1β production [237]. In addition, TNF upregulates ROS, which triggers the activation of caspase-1, thus inducing IL-1β release in response to TNF stimulation [237]. The macrophages we used were differentiated by M-CSF, whereas other studies used cell lines or macrophages derived by GM-CSF [237]. This chapter shows the similar expression of elements in NLRP3 inflammasome (caspase-1, NLRP3). However, the increased expression of pro-IL-1β in macrophages from TNF deficient mice suggests the high priming efficiency of LPS.

In summary, TNF deficiency results in alternative activation of splenic macrophages following *L. monocytogenes* infection. The inhibition of TNF in AAM differentiation might explain the essential role of TNF in defense against bacterial infection, and it ensures appropriate pro-inflammatory response to infection. The AAM differentiation in the absence of TNF leads to inappropriate immune responses and tissue damage following *L. monocytogenes* infection.

# Chapter 4

## Role of TNF in alternative activation of peritoneal macrophages following *L. monocytogenes* infection

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## 4.1 Introduction

TNF is an important inflammatory cytokine that is essential in regulating immune responses to bacterial infection. Macrophages are the main cell population that kill *L. monocytogenes* in the early stages of infection [238]. Macrophages reside in almost every tissue such as spleen, liver, and peritoneum [38]. They are the essential innate immune cells in phagocytosis of bacteria, production of cytokines, and activation of T cells [38]. Following inflammation with infections such as *L. monocytogenes*, bone marrow derived monocytes migrate into infected tissue and differentiate into TNF and inducible nitric oxide synthase (iNOS) producing dendritic cells (TipDCs) [69]. These cells are essential in mediating immune responses to *L. monocytogenes.* TNF combines with IFNγ, which activates macrophages into classically activated macrophages (CAM) [110]. Conversely, in response to a different microenvironment, macrophages activated by IL-4 or IL-13 differentiate into alternatively activated macrophages (AAMs) [110]. AAMs are anti-inflammatory therefore important for tissue repair and wound healing by producing anti-inflammatory cytokines (TGF-β, IL-10) [110]. The surface markers CD206, TGM2 and Fizz1 are upregulated in AAMs [98]. In addition, the enzyme Arginase 1(Arg1) is upregulated and essential for AAM metabolism [98]. Arg1 and iNOS competitively use the same L-arginine substrate. Arg1 converts L-arginine into L-ornithine whereas iNOS uses L-arginine to produce nitric oxide [239]. As an important pro-inflammatory cytokine, it has been suggested that TNF inhibits macrophage differentiate into AAMs in tumours [34] and in the parasite *Leishmania major* model [35]. We therefore hypothesised that TNF also inhibits AAM differentiation during infection with *L. monocytogenes*.

Peritoneal macrophages reside in the peritoneal cavity which also includes B cells, T cells, NK cells, eosinophils and dendritic cells [1]. There are two subsets of peritoneal macrophages, large peritoneal macrophages (LPMs) and small peritoneal macrophages (SPMs) [1]. LPMs are F4/80$^{high}$CD11b$^{+}$ and SPMs are characterized as F4/80$^{low}$CD11b$^{+}$ [49]. CD11b is expressed on macrophages, eosinophils, T cells, B cells and it is required for adhesion with other immune cells [51]. F4/80 has been used as a marker for macrophages. It is expressed by peritoneal macrophages but exhibits at low levels in splenic macrophages [1]. Under steady state conditions, LPMs are the most abundant of the peritoneal macrophages [49]. After

inflammatory stimuli, peritoneal cell subsets are dramatically altered, including inflammatory monocytes which have migrated *en masse* from bone marrow. This results in increased SPMs, but LPMs disappear which is referred as 'macrophage disappearance reaction' [1]. SPMs and LPMs display distinct phagocytic abilities and levels of NO production in response to stimulation [49]. In response to LPS stimulation *in vitro*, SPMs produce enhanced levels of NO and TNF [240]. Under inflammatory conditions SPMs are differentiated from bone marrow derived monocytes, whereas LPMs renew by self-proliferation [1]. The proliferation of LPM correlates with tissue repair after inflammation [57], and Ki67 has been used as a marker of peritoneal macrophage proliferation [241].

The role of TNF in the alternative activation of peritoneal macrophage is unknown. In this study, we compared peritoneal macrophage and monocyte subsets in B6.WT and B6.TNF$^{-/-}$ mice during *L. monocytogenes* infection. The expression of CAM and AAM markers were studied in the investigation of TNF activity in peritoneal macrophage differentiation. We showed that TNF deficient peritoneal macrophages express high levels of Arg1 following *L. monocytogenes* infection, suggesting an AAM bias in the absence of TNF.

## 4.2 Results

### 4.2.1 TNF deficiency alters the peritoneal cellular composition during *L. monocytogenes* infection.

In order to investigate the role of TNF in peritoneal macrophage differentiation with *L. monocytogenes* infection, we first examined the TNF effects on peritoneal cellular composition after infection. The cell subsets in peritoneum during *L. monocytogenes* infection were compared between B6.WT and B6.TNF$^{-/-}$ mice. The total numbers of peritoneal cells in B6.WT and B6.TNF$^{-/-}$ mice were similar, though slightly higher in B6.TNF$^{-/-}$ mice (Fig 4.2.1A). Neutrophils migrate into infection sites rapidly following *L. monocytogenes* infection, which is essential in the early control of bacterial infection [242]. At day 1 post infection, the abundance of neutrophils increased in the peritoneum in both strains of mice, indicating the initiation of infection. At day 3 and day 4, neutrophil abundance had returned to the basal levels in B6.WT mice, but remained elevated in B6.TNF$^{-/-}$ group (Fig 4.2.1B).

During inflammation in the peritoneum, the resident macrophage numbers are reduced, which has been referred to as the 'macrophage disappearance reaction' [54]. Following the infection

of *L. monocytogenes*, total peritoneal macrophages from B6.WT and B6.TNF$^{-/-}$ mice were decreased (Fig 4.2.1C). B6.TNF$^{-/-}$ mice harbored fewer peritoneal macrophages than wild-type controls at day 3 and day 4 post infection (Fig 4.2.1C). The number of monocytes increased at day 1 with *L. monocytogenes* infection in both strains of mice (Fig 4.2.1D). Peritoneal monocyte numbers in B6.TNF$^{-/-}$ mice were slightly higher than wild-type controls and were significantly higher at day 4 post infection (Fig 4.2.1D).

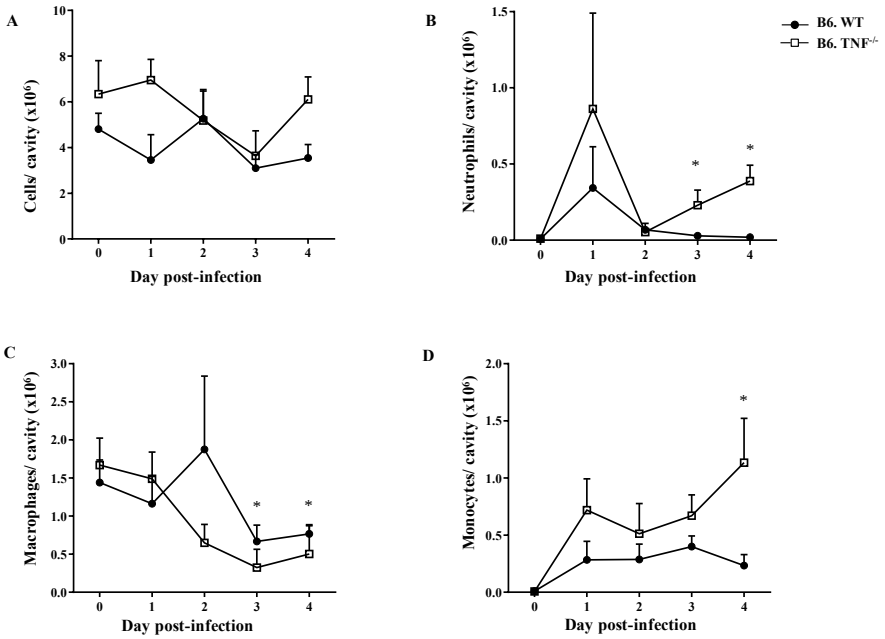


**Figure 4.2.1: The subsets of peritoneal neutrophil, macrophage, monocyte with *L. monocytogenes* infection.** B6.WT mice and B6.TNF$^{-/-}$ mice were infected with 1,000 *L. monocytogenes* intraperitoneally. The total number of peritoneal cells after infection at indicated days was analysed with flow cytometry. Total number of peritoneal cells (A), neutrophils (B), macrophages (C) and monocytes (D) from non-infected and *L. monocytogenes* infected B6.WT and B6.TNF$^{-/-}$ mice. n = 4-8 mice from two independent experiments, error bars represent SEM, *$p < 0.05$.

### 4.2.2 SPM and LPM in B6.WT and B6.TNF$^{-/-}$ mice showed different kinetics following *L. monocytogenes* infection.

We have shown that peritoneal macrophages in B6.TNF$^{-/-}$ mice decreased at day 3 and day 4 post infection compared to B6.WT mice. We then investigated the kinetics of SPM and LPM during *L. monocytogenes* infection. LPMs (F4/80$^{high}$CD11b$^{+}$) were the more abundant subset under naïve and *L. monocytogenes* infected conditions in both strains of mice (Fig 4.2.2A). The numbers of SPM (F4/80$^{low}$CD11b$^{+}$) in B6.WT and B6.TNF$^{-/-}$ mice were elevated at day 1 post infection. SPM numbers were increased in B6.WT whereas reduced in B6.TNF$^{-/-}$ mice at day

2 post infection, then SPMs were disappeared in both strains at day 3 and day 4 post infection (Fig 4.2.2B). The difference reached significance after infection for 4 days. In addition, LPM numbers in B6.TNF$^{-/-}$ mice were significantly reduced at day 3 and day 4 after infection, compared with wild-type controls (Fig 4.2.2C).

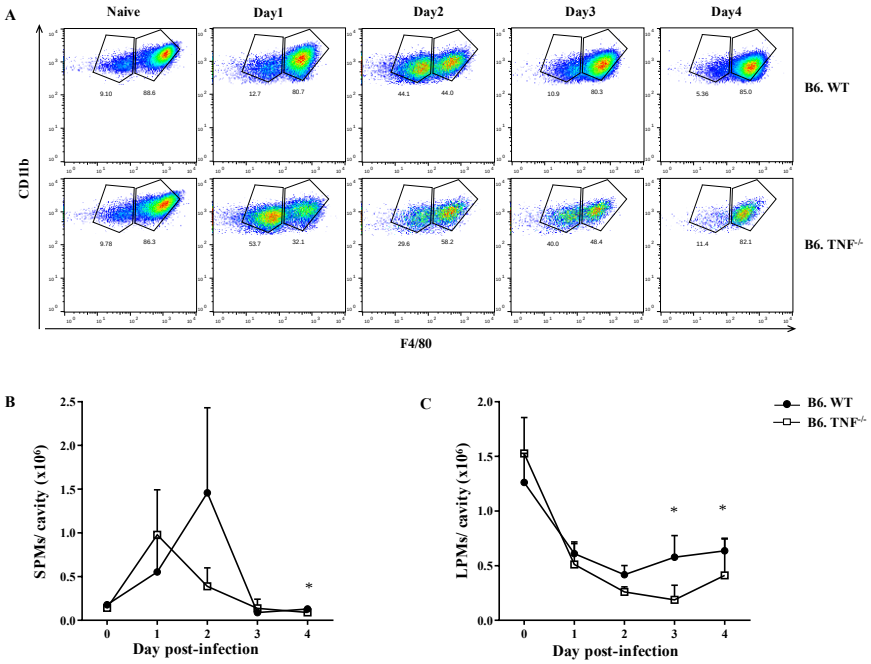


**Figure 4.2.2: The kinetics of SPM and LPM subset in peritoneal macrophages.** B6.WT and B6.TNF$^{-/-}$ mice were infected with 1,000 *L. monocytogenes* intraperitoneally. SPMs and LPMs were analysed using flow cytometry. Representative plots of peritoneal macrophages were identified as SPMs and LPMs according to the expressions of F4/80 and CD11b (A). The numbers of SPM (B) and LPM (C) were analysed. n = 4-8 mice from two independent experiments, error bars represent SEM, *$p < 0.05$.

### 4.2.3 Phenotypes of peritoneal macrophage and monocyte following *L. monocytogenes* infection.

The expression of CCR2 and MHC-II are important phenotypes of peritoneal macrophage and monocyte. We therefore investigated the levels of CCR2 and MHC-II expression in SPMs, LPMs and monocytes by flow cytometry. Monocytes and SPMs expressed higher levels of CCR2 in both strains of mice, suggesting the bone marrow derivation of monocytes and SPMs following *L. monocytogenes* infection (Fig 4.2.3A-C). There was no significant difference in the expression of CCR2 in SPMs and monocytes between B6.WT and B6.TNF$^{-/-}$ mice (Fig 4.2.3A, 4.2.3C). LPMs from B6.TNF$^{-/-}$ mice expressed increased levels of CCR2 than wild-

type controls at day 3 and day 4 post infection (Fig 4.2.3B). It has been described that SPMs express higher level of MHC-II than LPMs [49]. We showed higher expression of MHC-II in SPMs than LPMs in both strains of mice (Fig 4.2.3D-F). However, no significant differences were shown in the expression of MHC-II in SPMs, LPMs and monocytes (Fig 4.2.3E-F) between B6.WT and B6.TNF$^{-/-}$ mice.

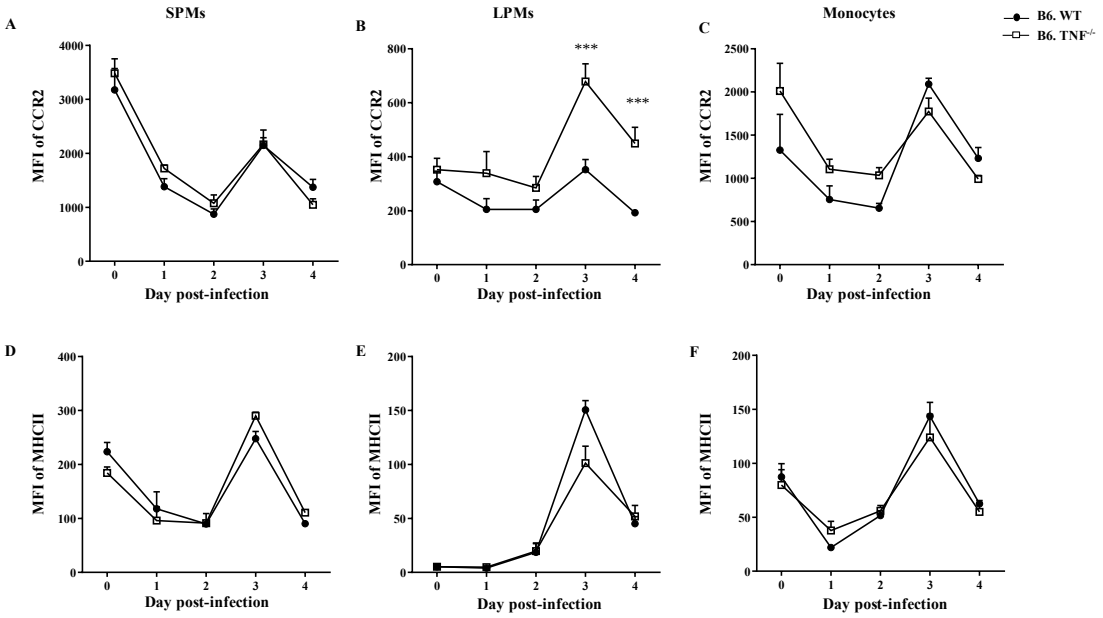


**Figure 4.2.3: Expression of CCR2 and MHC-II in SPM, LPM and monocyte.** B6.WT and B6.TNF-/- mice were intraperitoneally infected with 1,000 L. monocytogenes. Peritoneal cells were collected and the mean of fluorescence intensities (MFIs) of CCR2 and MHC-II in the SPMs, LPMs and monocytes were analysed by flow cytometry. n= 4-8 mice from two independent experiments, error bar represents SEM, *p < 0.05, ***p < 0.001.

### 4.2.4 qPCR analysis in peritoneal macrophages and monocytes during *L. monocytogenes* infection for 3 days.

Our results showed that TNF effects the kinetics of peritoneal macrophages and monocytes during *L. monocytogenes* infection (Fig 4.2.2). To gain a deeper insight, we sorted peritoneal macrophages, monocytes at day 3 post infection with *L. monocytogenes*. The expression of CAM and AAM markers such as iNOS, Arg1, CD206, Fizz1, IL-10, and IL-6 was analysed by qPCR. TNF deficiency did not alter the expression of Arg1, iNOS in peritoneal macrophages and monocytes (Fig 4.2.4B, 4.2.4C). B6.TNF$^{-/-}$ peritoneal macrophages expressed increased levels of TGM2 and decreased CD206, unchanged levels of Fizz1, IL-10 and IL-6 (Fig 4.2.4D-

G). It suggested that TNF deficiency altered the TGM2 and CD206 expression in peritoneal macrophages. We compared gene expressions between peritoneal macrophage and monocyte. There was no significant difference in the expression of Arg1 in peritoneal macrophages and monocytes (Fig 4.2.4B). B6.TNF$^{-/-}$ peritoneal macrophages expressed higher level of iNOS than monocytes, but not in B6.WT controls (Fig 4.2.4C). The levels of CD206, TGM2, Fizz1, IL-10, and IL-6 in peritoneal macrophages were enhanced in monocytes in both strains of mice (except TGM2 in B6.WT mice). This suggested the AAM bias in peritoneal macrophages after *L. monocytogenes* infection for 3 days in B6.WT and B6.TNF$^{-/-}$ mice.

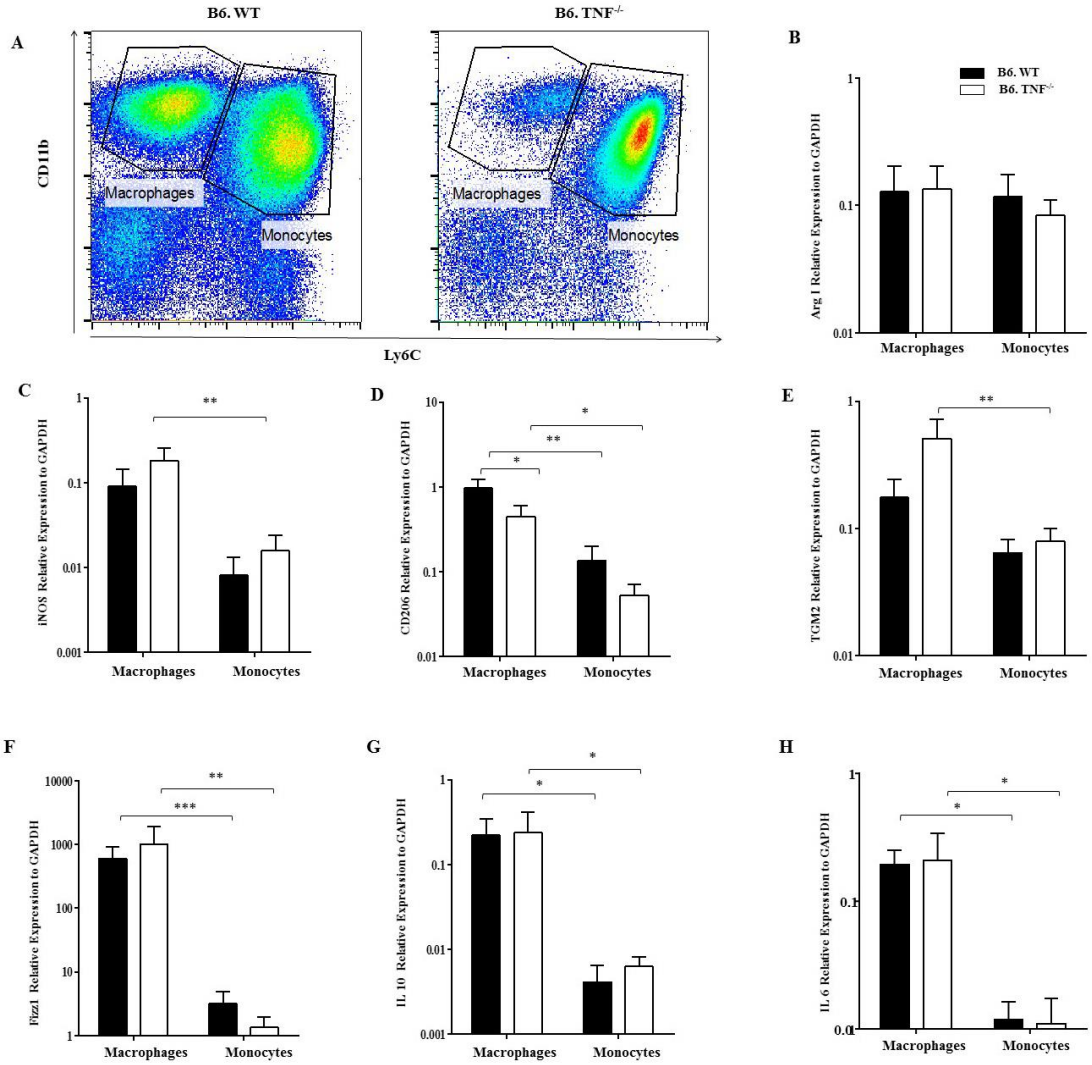

**Figure 4.2.4: Gene expression of macrophages and monocytes in the peritoneum at day 3 post infection.** B6.WT and B6.TNF$^{-/-}$ mice were infected with *L. monocytogenes* for 3 days. Peritoneal macrophages and monocytes were sorted using flow cytometry, gene expression was analysed by qPCR. Representative plots of peritoneal macrophages and monocytes for cell sorting (A). The expression of relative mRNA of Arg1, iNOS, CD206, TGM2, Fizz1, IL-10, and IL-6 in peritoneal macrophages and monocytes (B-H). n = 3-8 mice for each group, error bars were SEM, *p < 0.05, **p < 0.01, ***p < 0.001.

### 4.2.5 Intact iNOS expression in peritoneal macrophages and monocytes from B6.TNF$^{-/-}$ mice after *L. monocytogenes* infection for 3 days.

Since iNOS is a key marker of CAM, we investigated the expression of iNOS in peritoneal macrophages and monocytes from B6.WT and B6.TNF$^{-/-}$ mice. The mean fluorescence intensity (MFI) of iNOS in SPMs, LPMs and monocytes was analysed using intracellular flow cytometry (Fig 4.2.5A). The iNOS expressions in SPM, LPM and peritoneal monocyte were similar in TNF deficient mice, compared with wild type controls (Fig 4.2.5B). LPMs, but not SPMs or monocytes, are the major iNOS producer in B6.WT and B6.TNF$^{-/-}$ mice (Fig 4.2.5B). Total numbers of iNOS$^{+}$ subsets (SPMs, LPMs and monocytes) were not significantly different (data not shown), iNOS$^{+}$ SPMs and LPMs were pooled into macrophages. The total numbers of iNOS producing peritoneal macrophages and monocytes were decreased in B6.TNF$^{-/-}$ mice, though did not reach significant difference (Fig 4.2.5C).

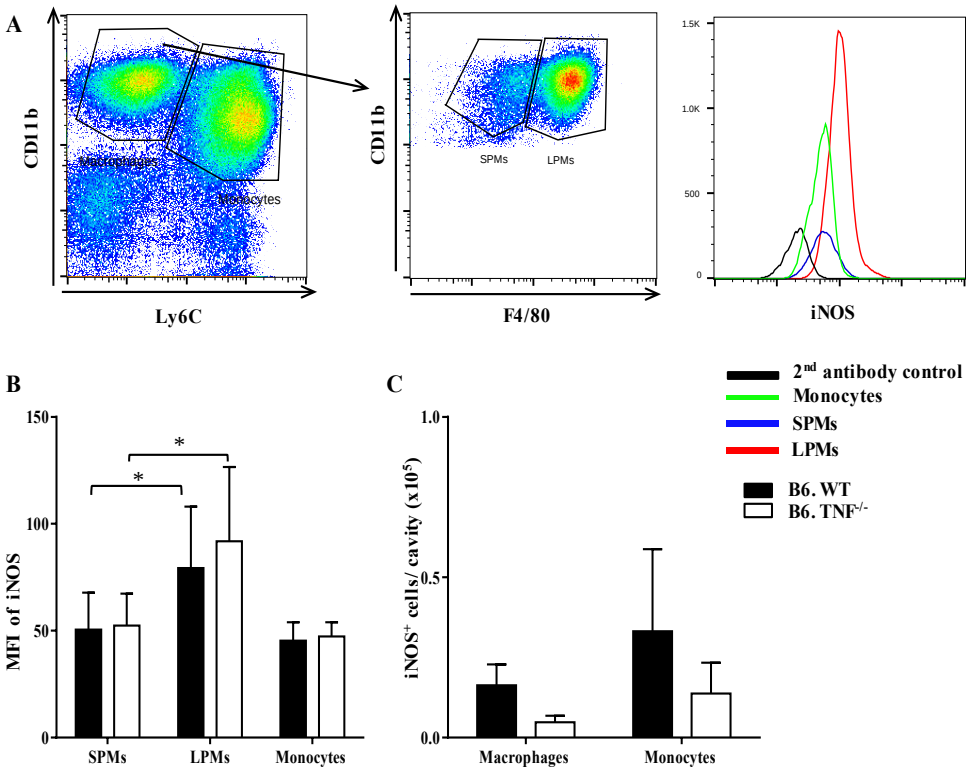


**Figure 4.2.5: iNOS expressions in peritoneal macrophage and monocyte after infection with *L. monocytogenes* for 3 days.** B6.WT and B6.TNF$^{-/-}$ mice were infected with 1,000 *L. monocytogenes* for 3 days. Expressions of iNOS in peritoneal macrophage and peritoneal monocyte were assessed using flow cytometry. Representative plots of iNOS expression in peritoneal macrophages, monocytes from B6.WT mice (A). MFI of iNOS in LPMs, SPMs and peritoneal monocytes (B). Total numbers of iNOS$^{+}$ peritoneal macrophages and monocytes in peritoneum cavity (C). n = 8 mice from two independent experiments, error bars indicate SEM, *p < 0.05.

### 4.2.6 Increased expression of Arg1 in LPMs from B6.TNF$^{-/-}$ mice during *L. monocytogenes* infection.

The high expression of Arg1 is an indicative phenotypic marker of AAM, so we investigated the Arg1 expression in SPMs, LPMs and monocytes by intracellular flow cytometry (Fig 4.2.6A). In both strains of mice, LPM was the more abundant subset expressing Arg1 with *L. monocytogenes* infection, displaying higher level of Arg1 than SPMs and monocytes (Fig 4.2.6B). In addition, total numbers of Arg1$^{+}$ subsets (SPMs, LPMs and monocytes) were not significantly different (data not shown), so SPMs and LPMs were pooled into macrophages. LPMs in the TNF deficient mice expressed increased Arg1 compared to wild type controls (Fig

4.2.6B). The total number of Arg1$^{+}$ peritoneal macrophages was higher in B6.WT mice (Fig 4.2.6C).

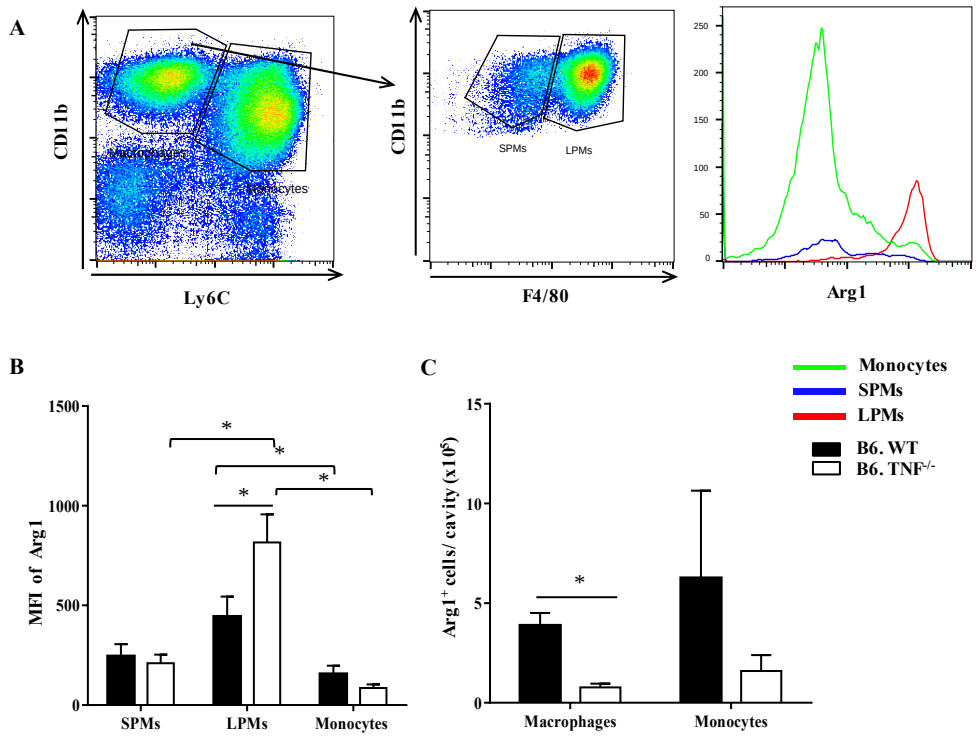


**Figure 4.2.6: Arg1 expression in the peritoneal macrophages and monocytes after infection with *L. monocytogenes* for 3 days.** B6.WT and B6.TNF$^{-/-}$ mice were infected with 1,000 *L. monocytogenes* for 3 days. Expression of Arg1 in peritoneal macrophages and monocytes was examined with intracellular flow cytometry. Representative plots of Arg1 expression in the peritoneal macrophages and monocytes in B6.WT mice (A). MFI of Arg1 in SPMs, LPMs and monocytes (B). Total numbers of Arg1$^{+}$ peritoneal macrophages and monocytes in peritoneum were shown (C). n = 8 mice from two independent experiments, error bars indicate SEM, *p < 0.05.

### 4.2.7 Normal cell proliferation in peritoneal macrophages in B6.TNF$^{-/-}$ mice with *L. monocytogenes* infection.

The high expression of Arg1 in LPMs from B6.TNF$^{-/-}$ mice suggests tissue repair after infection with *L. monocytogenes* for 3 days. We then investigated local proliferation in peritoneal macrophages, which is a characteristic of tissue repair. Ki67 has been used as a marker of cell proliferation and can be assessed using intracellular flow cytometry (Fig 4.2.7A). The levels of Ki67 in SPMs, LPMs and peritoneal monocytes were similar in B6.WT and B6.TNF$^{-/-}$ mice,

suggesting the peritoneal macrophage proliferation is independent of TNF (Fig 4.2.7B). The expression of Ki67 in LPMs were slightly higher than SPMs and significantly higher than monocytes (Fig 4.2.7B). Total numbers of $Ki67^+$ subsets (SPMs, LPMs and monocytes) were not significantly different (data not shown), so SPMs and LPMs were pooled into macrophages. The total numbers of $Ki67^+$ peritoneal macrophages were decreased in B6.$TNF^{-/-}$ mice, compared with wild-type controls (Fig 4.2.7C).

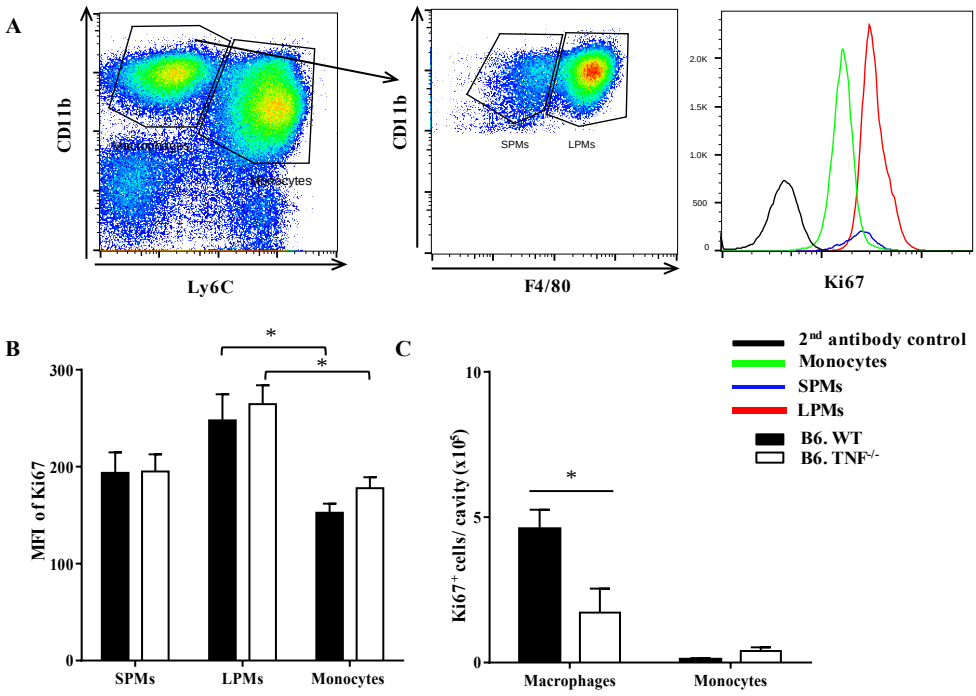


**Figure 4.2.7: Ki67 expression in peritoneal macrophages and monocytes following *L. monocytogenes* infection for 3 days.** B6.WT and B6.$TNF^{-/-}$ mice were infected with 1,000 *L. monocytogenes* for 3 days. Ki67 expression was assessed using intracellular flow cytometry. Representative plots of Ki67 expression in peritoneal macrophages and monocytes in B6.WT mice (A). MFI of Ki67 in SPMs, LPMs and peritoneal monocytes (B). Total numbers of $Ki67^+$ peritoneal macrophage and monocyte were analysed (C). n = 8 mice from two independent experiments, error bars indicate SEM, *p < 0.05.

## 4.3 Discussion

TNF is an important cytokine in defence against bacterial infection as TNFR1 deficient mice are susceptible to *L. monocytogenes* infection [31]. This chapter showed that in the absence of TNF, infection with *L. monocytogenes* caused peritoneal macrophages to differentiate into AAMs. The high expression of Arg1 in AAM impairs NO release, which would account for an increased susceptibility to infection. A similar situation has been demonstrated with *L. major*

infection [35]. Furthermore, the inhibition of TNF in AAM differentiation has also been demonstrated in tumour models [34]. Given this consistency, TNF appears essential in regulating macrophage function during infection.

Infections cause peritoneal inflammation result in a depletion of macrophages, which is referred as 'macrophage disappearance reaction' [54]. This was also observed in both B6.WT and B6.TNF$^{-/-}$ mice with *L. monocytogenes* infection. In the absence of TNF the reduction of macrophages was increased. This is correlated to a larger infiltration of monocytes into the peritoneum, thus TNF may have a role in attempting to maintain homeostasis of macrophages, but under severe inflammatory conditions it is overwhelmed by the infection. In addition, monocyte migration to infected tissue during infection requires the chemokine receptor CCR2 [182]. Results in this chapter showed that CCR2 expression in LPMs is increased in the absence of TNF. This is consistent with research showing that exogenous TNF reduces the expression of CCR2 [243]. The increased expression of CCR2 appears to lead to B6.TNF$^{-/-}$ LPMs migration out of the peritoneal cavity. LPMs have been indicated to migrate from peritoneum to omentum after intraperitoneal injection of black carbon particles [55]. The extensive migration of B6.TNF$^{-/-}$ LPMs might explain the reduced number of LPMs following *L. monocytogenes* infection.

Following the infection with *L. monocytogenes*, TNF absence leads to change of the peritoneal macrophage phenotype. qPCR analysis in the expression of Arg1 in peritoneal macrophages did not show significant difference between B6.WT and B6.TNF$^{-/-}$ groups. However, the higher expression of Arg1 in LPMs was found in B6.TNF$^{-/-}$ mice using flow cytometry. In order to investigate the expression of Arg1 at transcriptional level, LPMs will have to be isolated in future experiments. LPMs might be the subset of peritoneal macrophages which display distinct phenotypes in the absence of TNF. As an essential murine marker of CAM, iNOS showed a similar expression in B6.WT and B6.TNF$^{-/-}$ peritoneal macrophages. The intact iNOS expression in B6.TNF$^{-/-}$ mice has also been demonstrated in the *L. major* infection model [35]. In addition, our data showed that TNF-deficient peritoneal macrophages have increased Arg1, TGM2 and decreased CD206 expression, but unchanged Fizz1 and IL-10 expression. *Schleicher et.al* had reported the upregulated expression of Arg1, CD206 and similar expression of Fizz1 in draining lymph nodes macrophages from TNF$^{-/-}$ mice during *L. major* infection [35]. IL-10 expression is regulated by transcription factor- cAMP- responsive element binding protein (CREB), our data may suggest that TNF signalling is not required in the

activation of CREB during *L. monocytogenes* infection [244]. The distinct phenotype of 'AAMs' from $TNF^{-/-}$ mice may occur during bacterial and parasite infection. The mechanism under the role of TNF in AAM phenotypes with *L. monocytogenes* infection needs to be further understood. During the infection of *L. monocytogenes*, the 'AAMs' with increased expression of Arg1, TGM2 and decreased CD206 might not 'typical AAMs'. The expression of Arg1 and CD206 is regulated by transcription factor STAT6 [245], TNF in enhancing CD206 expression during *L. monocytogenes* infection is required to be investigated further. Additionally, it has been indicated that high expression of Arg1 impairs NO release in the site of infection, which causes non-healing infection with *L. major* [35]. The high level of Arg1 in 'untypical AAMs' in B6.$TNF^{-/-}$ mice might responsible to high susceptibility to *L. monocytogenes* infection.

Following infection, monocytes are recruited from bone marrow to the infected site (e.g. peritoneal cavity). SPM is the peritoneal macrophage subset differentiated from bone marrow derived monocytes. LPMs are considered to be resident macrophages that are maintained by self-proliferation. The higher expression of CCR2 in SPM, but not in LPMs suggests the bone marrow origin of SPM.

Ki67 is the marker of the cell cycle and it is absent in resting cells ($G_0$ phase) but present in the active phases of cell cycle [246]. Macrophage proliferation is a phenotype of IL-4 induced Th2 inflammation [57]. *L. monocytogenes* induced macrophage proliferation correlates with the AAM phenotype in macrophages [247]. It allows the liver to return to homeostasis after bacterial infection. The zinc finger transcription factor GATA-binding protein 6 (GATA6) appears to be involved in proliferation related gene profile of peritoneal macrophages [67]. However, loss of TNF signalling did not alter the proliferation of LPM during *L. monocytogenes* infection as a similar level of Ki67 was found in B6.$TNF^{-/-}$ mice. It may suggest that TNF is not required in GATA6 regulated proliferation in peritoneal macrophages. In response to the infection of *L. monocytogenes*, a higher level of Ki67 was found in LPM but not in SPM. It could suggest that proliferation of LPM occurs during *L. monocytogenes* infection as has been indicated a high Ki67 expression in liver resident macrophages following the infection of *L. monocytogenes* [247].

Neutrophils are the essential immune cells in killing *L. monocytogenes*. The absence of TNF leads to elevated neutrophil numbers in the peritoneal cavity after day 3 infection. TNF has been indicated to induce the expression of CXCL1 (KC) and CXCL2 (MIP-2) [230], which facilitates the homing of neutrophils into infected tissues. The high expression of CXCL1 was

reported in the liver from TNF deficiency mice with *L. monocytogenes* infection, suggesting CXCL1 may be the chemokine responsible for infiltration of neutrophils in TNF deficiency [232]. Chemokines CXCL1 (KC) and CXCL2 (MIP-2) might mediate the TNF related neutrophil recruitment with *L. monocytogenes* infection. In addition, higher neutrophil number in the peritoneal cavity might lead to over inflammation in B6.TNF$^{-/-}$ mice. On the other hand, after the infection of *L. monocytogenes* for 3 days, peritoneal macrophages are characterized with AAM phenotypes. They displayed high levels of CD206, TGM2, Fizz1 and IL-10 in peritoneal macrophages in both B6.WT and B6.TNF$^{-/-}$ mice. The peritoneal macrophages with AAM phenotype are essential in mediating tissue repair and remodel. A decreased number of peritoneal macrophages in B6.TNF$^{-/-}$ mice appears to lead to ongoing inflammation during bacterial infection. It is in line with the increased neutrophil number in B6.TNF$^{-/-}$ mice, which might lead to tissue damage.

The inhibition of TNF in AAM differentiation is essential in TNF mediated pro-inflammatory responses. Appropriate control of TNF drives the appropriated response to infections, such as *L. monocytogenes*. Since anti-TNF therapy has been widely used in the treatment of inflammatory diseases, the high risk of bacterial infection needs to be considered in the condition of TNF absence.

# Chapter 5

## Role of TNF in macrophage phagocytosis of *L. monocytogenes* and tumour cells

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## 5.1 Introduction

Macrophages are a heterogeneous population of innate immune cells with the important role of protection against pathogens. Macrophage phagocytosis involves receptor recognition, cytoskeleton rearrangement and phagosome maturation [122]. During the process of phagocytosis pathogens are endocytosed and become engulfed as membrane coated phagosomes [248]. The phagosomes then fuse with lysosomes to form an acidic environment to degrade the pathogens [122]. Actin polymerization is required for pathogen uptake and reactive oxygen and nitrogen species contribute to the degradation of the pathogen [122]. TNF appears to inhibit the clearance of apoptotic cells from the lung by reducing phagocytic efficiency [249]. In a murine model of otitis media TNF was shown to have an important role in clearing bacteria during a middle ear infection. TNF deficient macrophages were found to have a reduced ability to endocytose nontypeable Haemophilus influenza [250]. Furthermore, TNF appears to enhance IFNγ primed macrophage phagocytosis of apoptotic cells in a nitric oxide (NO) dependent manner [251]. Therefore, the roles of TNF in macrophage phagocytosis under different activation states and with different target cells are unclear.

*L. monocytogenes* and Devil Facial Tumour Disease (DFTD) provide useful targets to investigate role of TNF in macrophage phagocytosis. *L. monocytogenes* provide a target for phagocytosis of bacteria. After being recognized by macrophages, *L. monocytogenes* are phagocytosed within a phagosome and escape into cytoplasm by disrupting the phagosome membrane [4]. Then, *L. monocytogenes* replicate in the cytoplasm and infect the neighbouring cells through actin-based motility [252]. Listeriolysin O (LLO) of *L. monocytogenes* is required in bacterial escape from the phagosome by disrupting the phagosome membrane [253]. Moreover, actin assembly-inducing protein (ActA) of *L. monocytogenes* plays a central role in listerial intracellular motility through promoting actin recruitment and polymerization [254].

Devil Facial Tumour Disease (DFTD) cells provide an example of cancer cells. They were selected for two main reasons. Firstly, to determine if DFTD cells could be phagocytosed and secondly to investigate the role of TNF in phagocytosis of tumour cells. DFTD was first recorded in 1996 and is responsible for the catastrophic decline of the Tasmanian devil (*Sarcophilus harrisii*) population [255]. The cancer is transmitted as an allograft by biting and

is characterized by tumours located on the face and neck of infected devils [191]. Histological and immunohistochemical analyses of lymphoid organs have shown that Tasmanian devils possess the components required for a competent immune system [202]. Changes in histone deacetylation results in an absence of MHC-I expression on the DFTD cell surface, thereby causing the cancer cells to escape $CD8^+$ T cell recognition [199]. Devil NK cells appears to have the capacity for cytotoxicity against DFTD cells in an antibody dependent manner [204]. It is unknown whether devil macrophage can phagocytosis DFTD cells, hence the selection of these cells for this study.

Macrophages from wild type and B6.$TNF^{-/-}$ mice were isolated and the phagocytosis of *L. monocytogenes* and DFTD cells were investigated. This chapter showed that DFTD cells can be phagocytosed by murine macrophages. After activation by IFNγ/LPS or LPS alone, TNF was found to reduce the efficiency of macrophage phagocytosis.

## 5.2 Results

### 5.2.1 Role of TNF in thioglycollate- elicited peritoneal macrophage phagocytosis of *L. monocytogenes*.

Thioglycollate-elicited peritoneal macrophages from B6.WT and B6.$TNF^{-/-}$ mice were used to investigate the role of TNF in macrophage phagocytosis of *L. monocytogenes.* Macrophages were infected with *L. monocytogenes* (MOI 10) and the phagocytic ability was analysed using fluorescence microscopy (Fig 5.2.1A). As shown in Fig 5.2.1B, in untreated or IFNγ/LPS activated macrophages, the number of *L. monocytogenes* in infected macrophages was similar for the B6.WT and B6.$TNF^{-/-}$ group. Following activation with IL-4, macrophages from B6.$TNF^{-/-}$ mice phagocytosed less *L. monocytogenes* than wild type controls (Fig 5.2.1B). But IL-4 activation did not show difference in percentage of infected macrophages in B6.WT and B6.$TNF^{-/-}$ group (Fig 5.2.1C). A smaller number of *L. monocytogenes* was detected in macrophages but the percentage of infection was unchanged in B6.$TNF^{-/-}$ macrophages, suggesting the alteration of phagocytosis ability due to the lack of TNF. It suggests that TNF might be required in enhanced phagocytosis of *L. monocytogenes* during the activation by IL-4. The percentage of infected macrophages was enhanced by IFNγ/LPS treatment. But the IFNγ/LPS treatment did not alter the percentage of infected macrophages from B6.WT and B6.$TNF^{-/-}$ mice (Fig 5.2.1C).

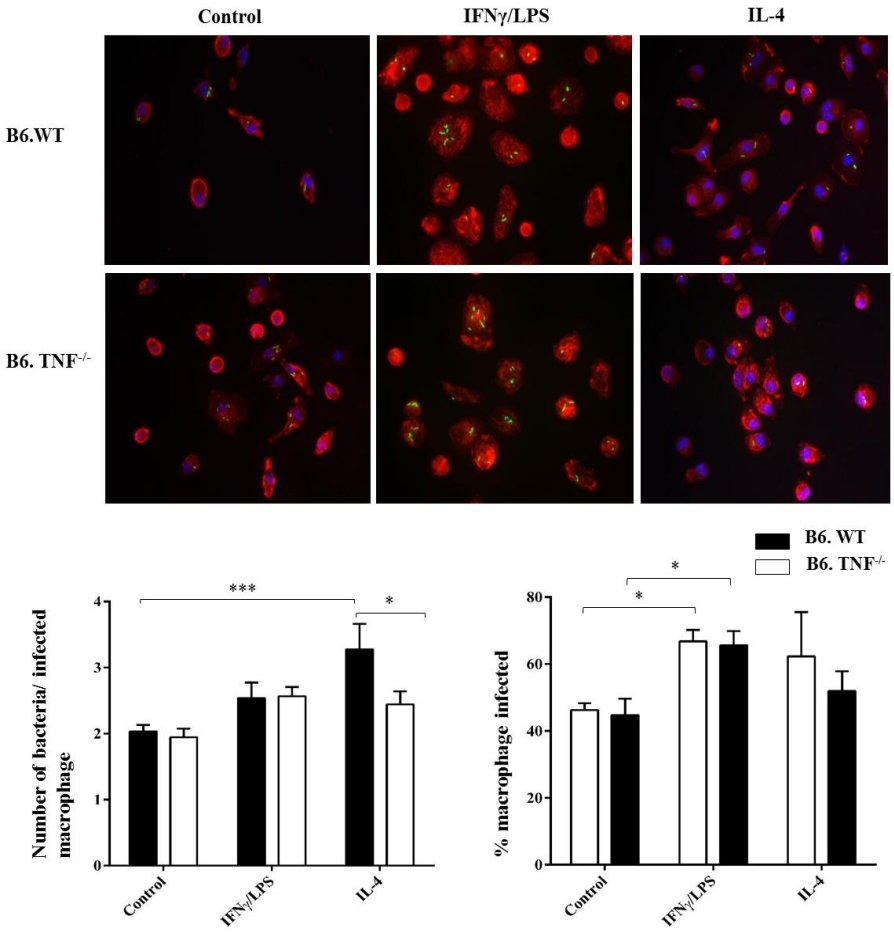


**Figure 5.2.1: Similar phagocytosis of *L. monocytogenes* in thioglycollate-elicited peritoneal macrophages from B6.WT and B6.TNF$^{-/-}$ mice.** Thioglycollate-elicited peritoneal macrophages from B6.WT and B6.TNF$^{-/-}$ mice were collected at day 3 after thioglycollate injection. Macrophages were activated with IFNγ and LPS or IL-4 as indicated in methods. Macrophages were infected with CFSE labelled *L. monocytogenes* at MOI 10 and phagocytosis capacity was analysed by fluorescence microscopy. (A) After infection with *L. monocytogenes*, cells were stained with anti-CD11b antibody and DAPI (Blue= DAPI; Red= macrophages; Green= *L. monocytogenes*). The number of *L. monocytogenes* in infected macrophages (B) and the percentage of phagocytosed macrophages was analysed (C) by fluorescence microscope. Data were from 4-6 mice from two or three independent experiments, error bars indicate SEM, * $p < 0.05$.

### 5.2.2 TNF is not involved in *L. monocytogenes* phagosomal escape in thioglycollate-elicited peritoneal macrophages.

After phagocytosis by macrophages, *L. monocytogenes* escape into cytosol to avoid degradation in phagosome [252]. The escaped *L. monocytogenes* move in the cytoplasm by actin polymerization [252], which is essential for *L. monocytogenes* intracellular survival. So,

we investigated the role of TNF in phagosomal escape from macrophages. The co-localization of phalloidin with *L. monocytogenes* represented *L. monocytogenes* that had escaped into the cytoplasm (Fig 5.2.2A). The phagosomal escape of *L. monocytogenes* in macrophages from B6.WT and B6.TNF$^{-/-}$ mice was analysed using confocal microscopy. Macrophages from B6.WT and B6.TNF$^{-/-}$ mice had similar percentages of phagosomal escape, in untreated, IFNγ/LPS or IL-4 activated macrophages (Fig 5.2.2B). Therefore, in the absence of TNF there is no increase in escape of *L. monocytogenes* into the cytoplasm. However, the percentage of *L. monocytogenes* that had escaped from the phagosome in macrophages from B6.WT and B6.TNF$^{-/-}$ mice was significantly less in IFNγ/LPS activated macrophages than control macrophages. Hence IFNγ/LPS activation may prevent escape into the cytoplasm. But no significant difference was found under the treatment of IL-4 (Fig 5.2.2B).

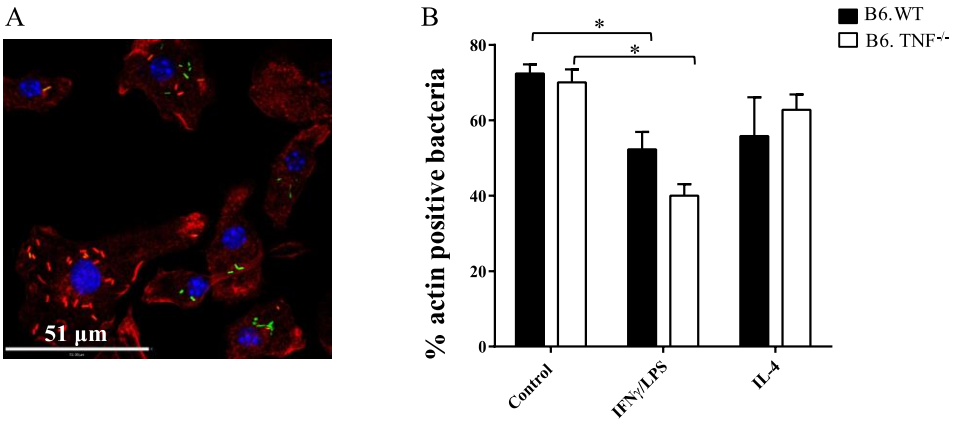


**Figure 5.2.2: TNF is not ionvolved in *L. monocytogenes* phagosomal escape in thioglycollate-elicited peritoneal macrophages.** B6.WT and B6.TNF$^{-/-}$ thioglycollate-elicited peritoneal macrophages were infected with CFSE-labelled *L. monocytogenes*. Phagosomal escape was analysed with confocal microscopy. (A) After infection of *L. monocytogenes*, cells were stained with phalloidin and DAPI (Blue=DAPI; Red=phalloidin; Green=*L. monocytogenes*). (B) Percentage of phalloidin positive CFSE-*L. monocytogenes*. Data were from 3-6 mice of two or three independent experiments, error bars indicate SEM, * $p < 0.05$.

### 5.2.3 DFTD cells can be phagocytosed by B6.WT and B6.TNF$^{-/-}$ BMDMs.

DFTD cells were used as cellular targets in the investigation of the role of TNF in macrophage phagocytosis of tumour cells. Due to the low yield of the thioglycollate-elicited peritoneal macrophages, we used BMDMs in the following experiments. To determine if DFTD cells can be phagocytosed, BMDMs from B6.WT and B6.TNF$^{-/-}$ mice (CTV-labelled; blue) were co-incubated with DFTD C5065 cells (CFSE-labelled; green). After 4 and 24 h culture, phagocytosis was analysed by confocal microscopy (Fig 5.2.3). Green DFTD cells could be identified within the blue macrophages from both B6.WT and B6.TNF$^{-/-}$ mice. When z-stacks were analysed the green fragments were identified inside the macrophages, suggesting the tumour cells had been engulfed (Fig 5.2.3). The degradation of phagocytosed cellular material involves the process of phagosome maturation through homotypic and heterotypic fusion of early endosomes, late endosomes and lysosomes [191]. The presence of lysosomal-associated membrane protein 1 (LAMP1) was used to discriminate a late phase of phagocytosis, after phagosome/lysosome fusion [256]. LAMP1 and engulfed tumour fragments were co-localized, confirming phagocytosis (Fig 5.2.3). As BMDMs from B6.WT and B6.TNF$^{-/-}$ mice can phagocytose DFTD cells, it suggests that TNF is not required for phagocytosis and that DFTD cells are not resistant to phagocytosis.

A B

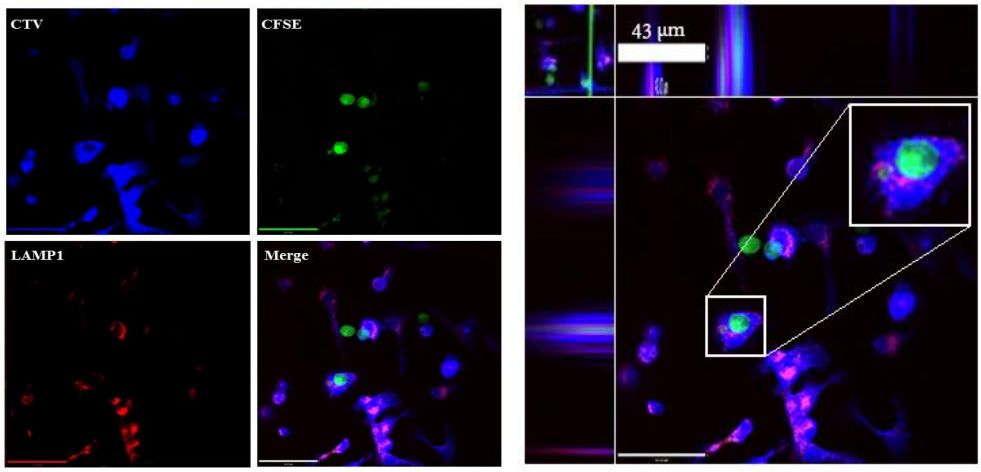


**Figure 5.2.3: Confocal microscope analysis of macrophage phagocytosis of DFTD cells.** CTV labelled BMDMs (blue) were incubated with CFSE labelled DFTD cells (green) for 4 h and 24 h, cells were fixed and permeabilized, then labelled with anti-LAMP1 antibody (red) (A). Phagocytosis was analysed by confocal microscopy. The z-stack demonstrated that tumour cells were inside B6.WT and B6.TNF$^{-/-}$ macrophages after 4 h and 24 h incubation. Arrows represent DFTD cells inside BMDMs (B).

### 5.2.4 TNF deficiency results in enhanced phagocytosis capacity in IFNγ/LPS treated macrophages.

Confocal microscopy showed that DFTD cells are phagocytosed by BMDMs from B6.WT and B6.TNF$^{-/-}$ mice, but to determine phagocytic efficiency flow cytometry was used. BMDMs were labelled with CTV and DFTD cells were labelled with CFSE, then incubated at ratios of 10:1 and 5:1 BMDMs: DFTD cells at 37 °C or 4 °C for 4 h. Phagocytosed DFTD cells were recognized as CFSE and CTV double positive cells. In the absence of stimulation (control) the phagocytic efficiencies of BMDMs from B6.WT and B6.TNF$^{-/-}$ mice were similar at both 10:1 (Fig 5.2.4A) and 5:1 (Fig 5.2.4B) ratios. It suggests that TNF is not required for macrophage phagocytosis of DFTD cells under steady-state conditions.

In order to determine TNF involvement in phagocytosis following macrophage activation, BMDMs were stimulated with IFNγ and LPS, IFNγ alone, LPS alone or IL-4 alone. Following IFNγ/LPS activation, BMDMs from B6.TNF$^{-/-}$ mice displayed enhanced phagocytosis efficiency at both 10:1 (Fig5.2.4A) and 5:1 (Fig 5.2.4B) ratios, compared with BMDMs from B6.WT mice. However, BMDMs from B6.TNF$^{-/-}$ mice stimulated with IFNγ, LPS or IL-4 alone

did not show a significant difference in phagocytic efficiency, compared with BMDMs from B6.WT mice (Fig 5.2.4A, 5.2.4B). To confirm that the CFSE and CTV double positive cells represented phagocytosis rather than cell binding, DFTD cells and macrophages were co-incubated at 4 °C for 4 h (Fig 5.2.4C, 5.2.4D). The proportion of double positive cells was substantially reduced providing support for phagocytosis at 37 °C rather than just binding to the cell surface.

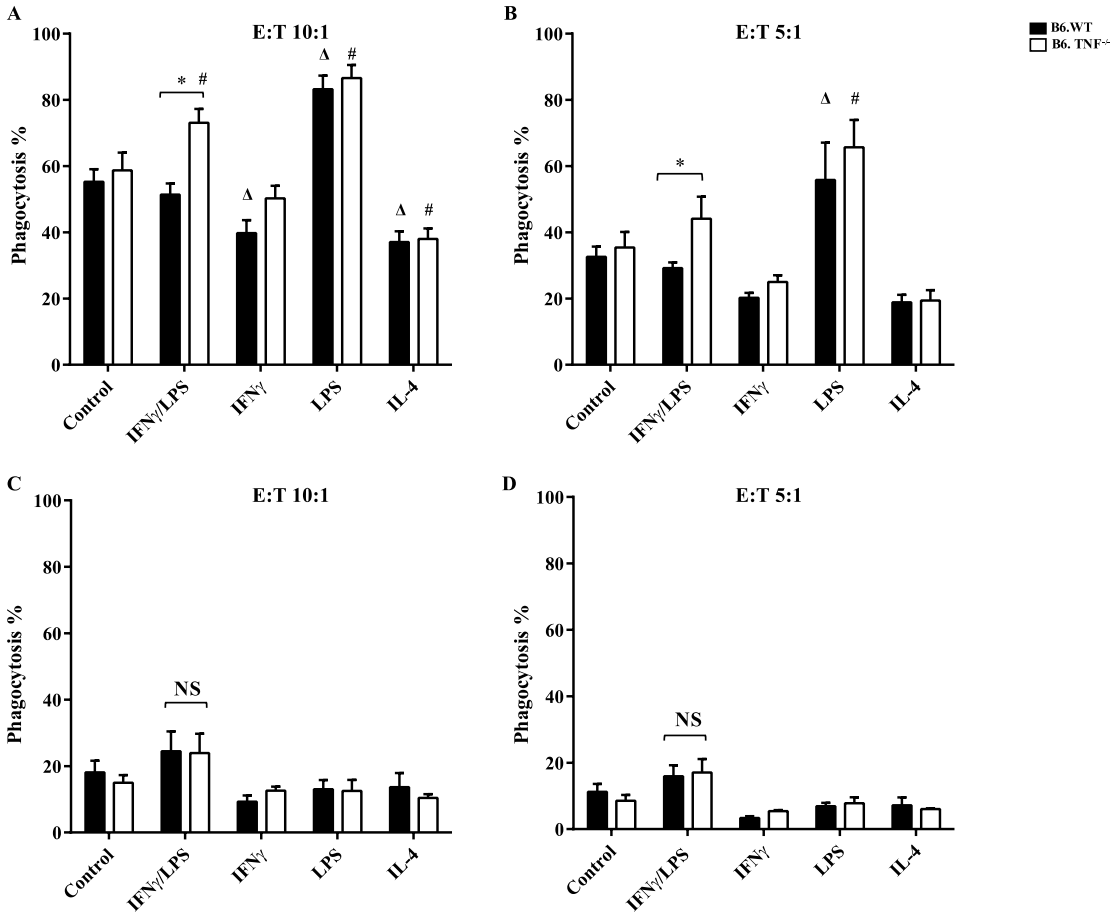


**Figure 5.2.4: IFNγ/LPS treated B6.TNF$^{-/-}$ BMDMs exhibited enhanced phagocytic efficiency of DFTD cells compared to B6.WT BMDMs.** BMDMs from B6.WT and B6.TNF$^{-/-}$ mice were treated with IFNγ/LPS, IFNγ alone, LPS alone, or IL-4 alone. BMDMs were labelled with CTV and DFTD cells were labelled with CFSE. After labelling, BMDMs were co-incubated with DFTD cells for 4 hours. The percentage of phagocytosed DFTD cells in the incubation of 37 °C (A, B) and 4 °C (C, D) was investigated by flow cytometry. Data were from 3-7 mice of two or three independent experiments, error bars indicate SEM, ΔP< 0.05 versus WT control, #P< 0.05 versus B6.TNF$^{-/-}$ control, *P< 0.05 versus WT versus B6.TNF$^{-/-}$, NS not significantly.

### 5.2.5 IFNγ/LPS, LPS activated BMDM from B6.TNF$^{-/-}$ mice phagocytose and kill DFTD cells more effectively than BMDM from B6.WT mice following 24 h incubation.

Macrophage phagocytosis and killing of DFTD cells was analysed with flow cytometry by enumerating the number of viable DFTD cells remaining. The absolute number of free DFTD cells was calculated using cell count beads. IFNγ/LPS and LPS alone activated BMDMs from B6.TNF$^{-/-}$ mice cultured with DFTD cells for 24 h resulted in fewer DFTD cells remaining compared to BMDMs from B6.WT mice at 10:1 and 5:1 ratios (Fig 5.2.5A, 5.2.5B). However, IFNγ or IL-4 activated BMDMs from B6.TNF$^{-/-}$ mice had similar number of DFTD cells with wild-type controls at 10:1 and 5:1 ratios (Fig 5.2.5A, 5.2.5B). These results indicate that LPS and potentially IFNγ/LPS activated macrophages from B6.TNF$^{-/-}$ mice phagocytosed more DFTD cells the macrophages from the B6.WT control mice. Thus, although phagocytosis by activated BMDMs was observed in the presence of TNF, the absence of TNF appeared to increase the phagocytic activity.

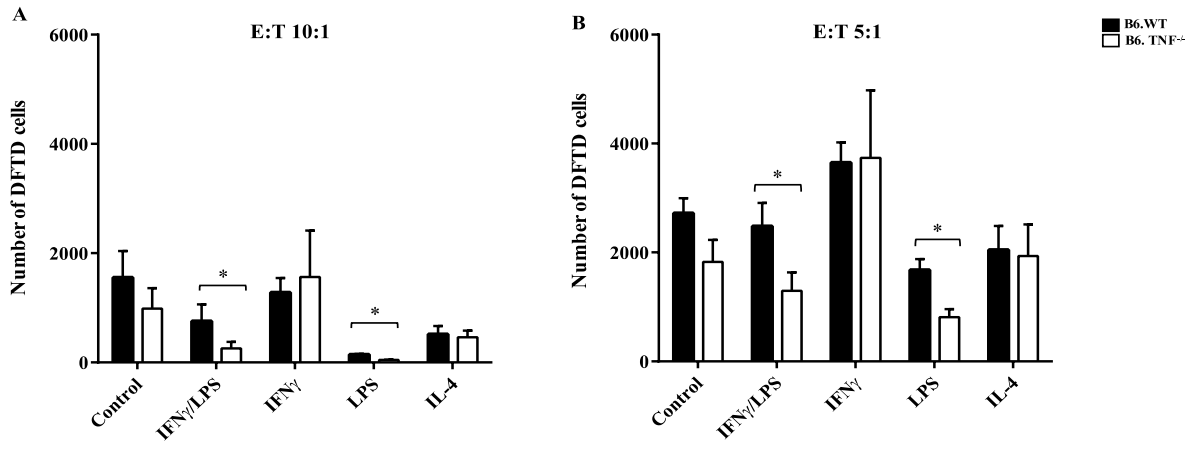


**Figure 5.2.5: IFNγ/LPS activated B6.TNF$^{-/-}$ macrophages remain less DFTD cells after 24 hours incubation.** After co-incubation with macrophages and DFTD cells for 24 hours, number of DFTD cells at (A) (E: T) 10:1, (B) (E: T) 5:1 were determined by flow cytometry by collecting all cells in each treatment. The numbers of DFTD cells were calculated as describing in materials and methods chapter. Data were from 3-5 mice of two or three independent experiments, error bars indicate SEM, *p <0.05.

### 5.2.6 Less nitric oxide is released from IFNγ/LPS, LPS activated B6.TNF$^{-/-}$ BMDMs after incubation with DFTD cells for 24 h.

Nitric oxide (NO) is an essential effector molecule in macrophages and is cytotoxic to tumour cells [91]. As TNF regulates the production of NO in macrophages [95], we investigated whether NO was reduced in the absence of TNF during phagocytosis of DFTD cells by IFNγ/LPS activated BMDMs. IFNγ/LPS, IFNγ alone, LPS alone treated BMDMs from B6.WT, B6.TNF$^{-/-}$ mice were incubated with DFTD cells for 24 h and the NO contents in the cell culture supernatant were measured. As shown in Figure 5.2.6, untreated BMDMs released undetectable NO and were similar in both genotypes. Compared with the B6.WT controls, less NO was released from IFNγ/LPS treated B6.TNF$^{-/-}$ BMDMs after incubation with DFTD cells for 24 h (Fig 5.2.6A, 5.2.6B). Similarly, less NO was released in LPS activated BMDMs from B6.TNF$^{-/-}$ mice than wild-type controls (Fig 5.2.6A, 5.2.6B). IFNγ treatment did not alter NO production of B6.WT, B6.TNF$^{-/-}$ BMDMs after incubation with DFTD cells for 24 h (Fig 5.2.6A, 5.2.6B).

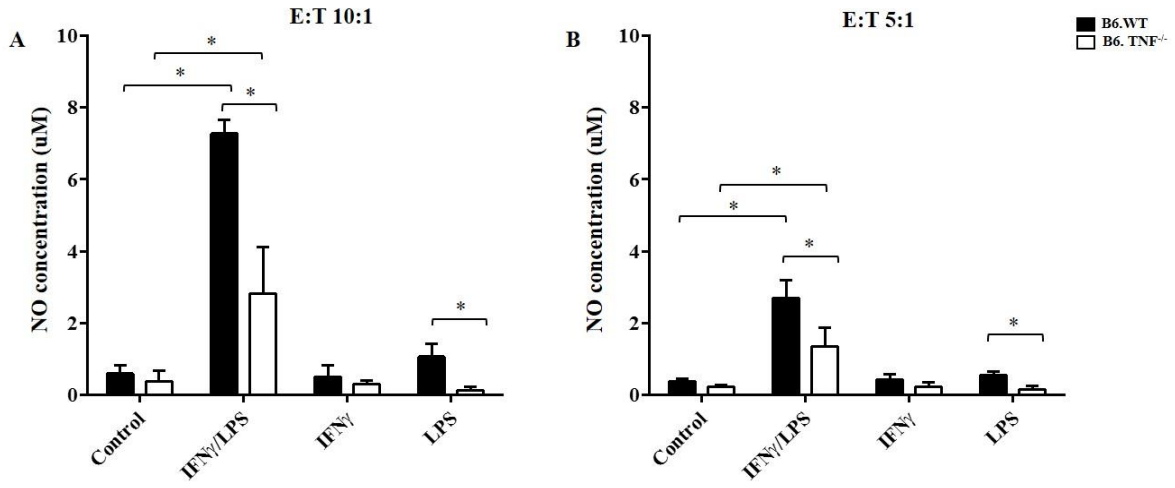


**Figure 5.2.6: Reduced NO production from IFNγ/LPS, LPS activated B6.TNF$^{-/-}$ BMDMs phagocytosis of DFTD cells.** B6.WT and B6.TNF$^{-/-}$ BMDMs were stimulated with IFNγ/LPS, IFNγ alone, or LPS alone. BMDMs and DFTD cells were co-incubated at E: T 10:1 (A) and 5:1 (B) for 24 h, the concentration of NO in the cell culture supernatant was analysed by Griess assay. Data were from 3 mice, error bars indicate SEM, * P<0.05, ** p < 0.01.

### 5.2.7 iNOS inhibitor cannot reverse enhanced phagocytic activity of IFNγ/LPS activated B6.TNF$^{-/-}$ macrophages.

To determine whether NO is required for phagocytosis in the presence of TNF, NO release was

blocked by the inhibitor of nitric oxide synthesis, L-N6- (1-Iminoethyl) lysine dihydrochlorideinducible (L-NIL). After incubation with IFNγ/LPS or IFNγ/LPS and L-NIL for 24 h, BMDM from B6.WT and B6.TNF$^{-/-}$ mice were co-incubated with DFTD cells for 24 h. The numbers of DFTD cells were measured by flow cytometry and cell counting beads. As shown in Figure 5.2.7, L-NIL did not alter phagocytosis following stimulation with IFNγ/LPS of BMDMs from B6.WT mice or B6.TNF$^{-/-}$ mice. In B6.WT mice, L-NIL treatment in IFNγ/LPS activated BMDMs showed similar phagocytosis with IFNγ/LPS activated BMDMs (Figure 5.2.7). It suggests that nitric oxide was unrelated to the TNF inhibition of macrophage phagocytosis of DFTD cells.

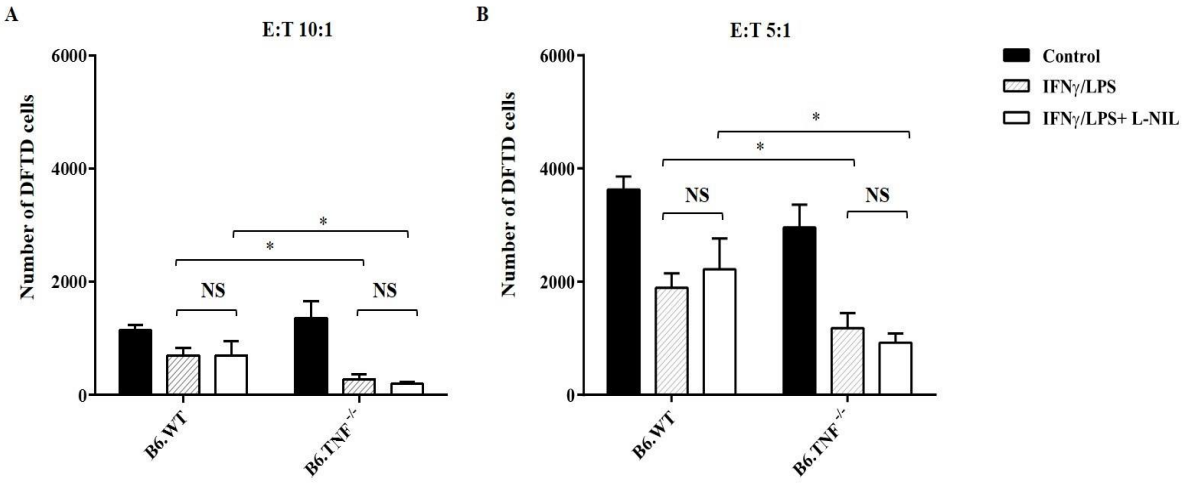


**Figure 5.2.7: NO is unrelated to TNF involved macrophage phagocytosis of DFTD cells.** B6.WT and B6.TNF$^{-/-}$ BMDMs were treated with IFNγ/LPS or IFNγ/LPS with L-NIL overnight. After incubation with BMDMs and DFTD cells for 24 hours, the numbers of DFTD cells at E: T 10:1 (A), 5:1 (B) ratios were analysed by flow cytometry and cell counting beads. The numbers of DFTD cells were calculated as methods described. Data were from 3-5 mice of two or three independent experiments, error bars indicate SEM, * P< 0.05, NS not significantly.

## 5.3 Discussion

Macrophage phagocytosis is essential in defence against pathogens. This chapter investigated TNF activity in macrophage phagocytosis using *L. monocytogenes* and DFTD cells as models of bacteria and tumour cells. In the study of phagocytosis with *L. monocytogenes*, IFNγ/LPS treatment of macrophages resulted in an increased phagocytosis efficiency and reduced phagosomal escape. It has been reported that IFNγ/LPS activation enhances macrophage

phagocytosis with *L. monocytogenes* and retains *L. monocytogenes* in phagosomes [257], which is in line with results in this thesis. TNF did not appear to have a role in macrophage phagocytosis or preventing phagosomal escape of *L. monocytogenes*. But treatment with IFNγ/LPS or LPS alone, TNF reduced macrophage phagocytosis of DFTD cells.

*L. monocytogenes* and DFTD cells had been used to investigate the role of TNF in macrophage phagocytosis. In response to the activation of IFNγ/LPS, TNF deficiency leads to higher efficiency in phagocytosis of DFTD cells, but not *L. monocytogenes*. There are two potential reasons for the different role of TNF in macrophage phagocytosis of *L. monocytogenes* and DFTD cells. Firstly, the role of TNF in macrophage phagocytosis is distinct in different phagocytosis targets. Macrophages appear to have distinct mechanisms in the phagocytosis of bacteria and tumour cells. Secondly, thioglycollate-elicited peritoneal macrophages were used in the investigation of TNF effects in macrophage phagocytosis of *L. monocytogenes*. The eliciting agent Brewer's thioglycollate broth has been used to increase the yield of macrophages from peritoneal cavity [258]. However, thioglycollate injection causes inflammation in the peritoneal cavity and monocytes are recruited from bone marrow and differentiate into small peritoneal macrophages (SPM) [49]. Therefore, the studies of thioglycollate-elicited peritoneal macrophage reflect the function of resident SPMs and monocyte derived SPMs [49]. The problem is directly comparing phagocytosis when two different macrophage sources were used. In addition, macrophages might change their physiology function in response to thioglycollate injection, as they have defective intracellular killing of microorganism [259]. Due to the limitation of thioglycollate-elicited peritoneal macrophages, macrophages derived bone marrow were used in the study of TNF effects in macrophage phagocytosis of DFTD cells. Therefore, the different sources of macrophages might result in distinct function of TNF in phagocytosis.

The absence of MHC-I expression on DFT1 cells prevents the activation $CD8^+$ T cell adaptive immune response [199]. This would prevent an adaptive cell mediated immune response. An enhancement of innate immune response to DFTD such as macrophage phagocytosis may provide a mechanism to promote anti-DFTD immune responses. This could be either natural, in the wild, or artificial, through vaccination. The evidence that immunised devils [260, 261] and some wild devils [200] can produce antibodies against DFT1 cells is in line with antibody mediated opsonisation of DFT1 cells and subsequent phagocytosis. For this to occur, DFT1 cells would need the capacity to be phagocytosed. Tumour cells can avoid phagocytosis by

expressing inhibitory molecules such as the ‘don't eat me signal’, which is part of the immune checkpoint inhibitory pathway, preventing phagocytosis [262]. RNA transcriptome analysis identified CD47 from devil mononuclear cells, which aligned closely with other species [263]. Hence there is the potential for DFT1 cells to express this molecule to avoid phagocytosis.

There are some difficulties in collecting devil macrophages for the analysis of DFTD phagocytosis. In the absence of monoclonal antibodies to identify such molecules and the unavailability of devil macrophages, we conducted phagocytosis of DFT1 cells using bone marrow derived mouse macrophages. The interpretation of DFTD phagocytosis by murine macrophages might provide evidence that DFTD cells can be phagocytosed by macrophages. If mouse derived macrophages could phagocytose DFT1 cells, then devil macrophages should also have this ability. But some of the usual ligand interactions may not operate across species. The CD47 ‘don’t eat me’ signal may not work from devil to mouse macrophages, but may work devil-devil preventing phagocytosis. We showed the phagocytosis of DFTD cells by mouse macrophages by two independent mechanisms, flow cytometry and confocal microscopy. The confocal microscopy, provided the additional evidence of destruction of the DFT1 tumour cells. The susceptibility of DFT1 cells to phagocytosis has at least two important implications. Firstly, wild devils that have the capacity to make antibodies against DFT1 cells could promote phagocytosis of antibody opsonised DFT1 cells [200]. Secondly, devil immunised with DFT1 cells that produce antibody [260] could also opsonise the DFT1 cells and target them for phagocytosis. In both situations, promotion of a specific immune response could follow due to the interaction of the macrophages and cytokines produced during phagocytosis. This could result in cytokines upregulating MHC-I [199] and the DFT1 cells becoming targets for CD8 T cells, as shown with immunotherapy [261].

TNF reduced macrophage phagocytosis of DFTD cells following activation with IFNγ/LPS or LPS. This is consistent with previous finding that exogenous TNF inhibits macrophage phagocytosis of apoptotic cells [264]. Furthermore, LPS induced TNF production has been shown to reduce macrophage phagocytosis of apoptotic neutrophils in an autocrine way [265, 266]. In contrast, inhibition by TNF in macrophage phagocytosis was not observed with human IgG-opsonized erythrocytes [266]. Nor has TNF been reported to be essential in the enhancement of macrophage phagocytosis of apoptotic cells [251]. Consequently the role of TNF in macrophage phagocytosis of tumour cells is not fully understood. Results in this thesis showing enhanced phagocytosis by B6.TNF$^{-/-}$ macrophages indicates that TNF is not required

for uptake of DFTD cells. As macrophages can produce TNF either by LPS or IFNγ/LPS activation, endogenous TNF production could increase TNF to inhibitory levels [94, 267]. IFNγ could activate macrophages to produce TNF only in the present of LPS [268]. Although from a different source, TNF production from IFNγ primed macrophages has been demonstrated [251, 269]. IFNγ/LPS treatment induces higher level of TNF production than LPS alone [267]. Unlike IFNγ/LPS treatment, LPS activated B6.TNF$^{-/-}$ BMDMs display enhanced phagocytic ability at 24 h incubation but not 4 h incubation. The high production of TNF from IFNγ/LPS activated macrophages may inhibit phagocytosis of DFTD cells. The production of TNF from LPS, IFNγ and IFNγ/LPS treated macrophages needs to be further evaluated.

Phagocytosis of pathogens by macrophages requires NO and TNF dependent NO production is required for the enhanced phagocytosis of apoptotic cells by IFNγ activated macrophages [251]. However, NO can impair phagocytosis of fluorescent particles by affecting cytoskeletal assembly and pseudopod formation [270]. TNF is definitely involved in NO production as it induces iNOS expression via NF-κB [94, 95]. Furthermore, the expression of iNOS in IFNγ/LPS activation macrophages requires TNF [93]. TNF dependent NO production is required for the enhanced phagocytosis of apoptotic cells by IFNγ activated macrophages [251]. We blocked NO release by using L-NIL, but inhibition of NO did not alter phagocytosis efficiency in IFNγ/LPS treated macrophages. It suggests the NO is independent of TNF related IFNγ/LPS treated macrophages. Moreover, actin polymerization is an essential event in the process of macrophage phagocytosis. It has been suggested that exogenous TNF decreases actin reorganization in J774 macrophages [271]. The involvement of actin and TNF inhibition of phagocytosis activity in IFNγ/LPS activated macrophages needs to be further investigated.

Macrophage phagocytosis plays an essential role in defence against tumour. Tumour cells express high levels of programmed cell death protein 1 (PD-1) to inhibit macrophage phagocytosis of tumour cells [272]. Monoclonal antibodies blocking PD-1 has been a notable therapy in patients with melanoma and colorectal cancer [272]. Similarly, the inhibition of TNF in macrophage phagocytosis of tumour cells appears to be essential in host's response to tumour. Understanding the mechanisms of TNF in macrophage phagocytosis of tumour cells may thus be a target for the therapy of cancer.

# Chapter 6

# Final discussion

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Roles of TNF in regulating immune system are complex and diverse. As a pro-inflammatory cytokine, TNF is implicated in the pathogenesis of chronic inflammatory diseases, such as rheumatoid arthritis, Crohn's disease, and psoriasis [273]. It has indicated that increased release of TNF at inflammatory sites drives disease pathology [11]. Consequently, pharmacological interventions of TNF by using TNF antagonists have been the standard care in several chronic inflammatory conditions including rheumatoid arthritis and inflammatory bowel disease [273]. However, despite the widely use of anti-TNF therapy, the increased recrudescence of latent infections including leishmaniasis and tuberculosis occurs in clinical application [8]. Some cases were reported in induction of psoriasis with anti-TNF agents in inflammatory bowel disease patients [274]. Therefore, further understanding the mechanisms and biology of TNF acts on immune response may provide insight into anti-TNF therapy in inflammatory diseases and provide basis for avoiding undesired latent or re-infection with pathogens.

TNF can be produced by many types of cells but is mainly produced by macrophages and T cells during infections [5]. TNF has both beneficial and detrimental effects after microbial exposure. Secretion of TNF is essential in resolution of various infection of pathogens. TNFR1 deficient mice are high susceptible to *L. monocytogenes* infection [31]. TNF deficiency leads to progressive cutaneous and visceral disease with the infection of *L. major* (FEBNI strain) in C57BL/J mice [223]. $TNF^{-/-}$ mice succumbed to respiratory failure after infection by replicable mycobacteria and TNF acts as a negative regulator of type 1 immune response during bacterial infection [275]. Excessive production of TNF is related to increased organ injury and mortality rate in the condition of endotoxemia, bacterial infection and abdominal sepsis [276]. These disconcordant outcomes failed to answer the question about the role of TNF in mediating immune protection and immune mediated pathology. In this thesis, I investigated the role of TNF in the context of experimental *L. monocytogenes* infection and revealed roles of TNF in regulating macrophage mediated immune responses to defence against this intracellular infection. During *L. monocytogenes* infection, I found the bias of alternative activation in macrophages in the absence of TNF (chapter 3, 4), and TNF is required in macrophages restrict the release of IL-1β (chapter 3). Additionally, I showed that TNF is potentially involved in downregulating activated macrophages phagocytosis of tumour cells (chapter 5).

### 6.1 TNF in dampening AAM differentiation following *L. monocytogenes* infection

Classically activated macrophages (CAM) and alternatively activated macrophages (AAM) are the two main macrophage phenotypes that develop in response to different stimuli which promote pathogen clearance and tissue repair respectively [41]. It has been reported that TNF inhibits AAM differentiation in experimental cutaneous leishmaniasis [35] and in tumour models [34]. The common activity of TNF in AAM differentiation in experimental *L. monocytogenes* infection is examined in this thesis. In spleen, it has been indicated that bone marrow derived monocytes differentiate into dendritic cells with high ability in producing iNOS and TNF (TipDCs) [69]. These TipDCs are $Ly6C^{hi}$ and share the similar characterization of CAMs [41]. The chemokine receptor CCR2 is required in monocyte recruitment as the monocyte recruitment is inhibited in CCR2 deficient mice during *L. monocytogenes* infection [182]. Therefore, the splenic inflammatory monocytes in this thesis were characterized with $Ly6C^{hi}CCR2^{+}$. In the context of *L. monocytogenes* infection, $Ly6C^{hi}CCR2^{+}$ monocytes from $B6.TNF^{-/-}$ mice displayed the phenotype of AAMs with upregulated Arg1, TGM2 expression (Figure 3.2.4). Similar to the results of splenic monocytes, alternative differentiation was found in peritoneal macrophages from $B6.TNF^{-/-}$ mice during *L. monocytogenes* infection. Large peritoneal macrophages (LPMs) and small peritoneal macrophages (SPMs) are two subsets of peritoneal macrophages with distinct expression levels of F4/80 [1]. The level of Arg1 in LPMs from $B6.TNF^{-/-}$ mice was higher than in wild type controls, which may suggest that TNF dampens AAM bias in peritoneal macrophages during *L. monocytogenes* infection (Figure 4.2.6).

The AAM differentiation in the absence of TNF is consistent with the studies in *L. major* [35] and with tumour models [34]. AAMs can be identified as producing Arg1, whereas CAMs produce iNOS [277]. Both enzymes are essential for the metabolism of L-arginine, and compete for its use [277]. TNF has been shown to inhibit AAM gene expression by downregulating the expression of genes triggered by IL-4 such as Arg1 and CD206 [35]. The inhibition by TNF of IL-4 induced AAM gene expression involves the suppression of the transcriptional factor STAT6 [35]. Arg1 expression in IL-4 triggered macrophages is dependent on H3 acetylation at the *Arg1* promoter [35]. The downregulation of TNF reduces Arg1 expression through inhibition of H3 acetylation [35]. During infection with *L. monocytogenes*, the results showed lack of TNF affects on Arg1 and TGM2, but not CD206, expression (Figure 3.2.4). The role of TNF in modulating CD206 expression during the *L. monocytogenes* infection needs to be further characterised. The inhibition of TNF AAM

differentiation appears to coincide with the high susceptibility to *L. monocytogenes* infection. TNF did not alter the levels of iNOS in splenic and peritoneal macrophages during *L. monocytogenes* infection (Figure 3.2.4-5, Figure 4.2.5). Despite the normal activity of iNOS, excessive expression of Arg1 appears to inhibit NO production during infection with *L. major*, presumably by competing for the same substrate, resulting in failure to control infection in B6.TNF$^{-/-}$ mice [35]. Additionally, splenic AAMs with high level of Arg1 harboured more *L. monocytogenes* in B6.TNF$^{-/-}$ mice (Figure 3.2.5). The involvement of Arg1 in regulating NO production during experimental *L. monocytogenes* infection is worthy of further investigation.

In addition, the splenic Ly6C$^{low}$ monocytes were found to be AAMs during *L. monocytogenes* infection for 3 days. The two subsets of splenic monocytes (Ly6C$^{hi}$ and Ly6C$^{low}$ monocytes) exhibit distinct function with Ly6C$^{hi}$ monocytes being pro-inflammatory and Ly6C$^{low}$ monocytes having anti-inflammatory activities [37]. In response to *L. monocytogenes* infection, Ly6C$^{hi}$ monocytes migrate rapidly from bone marrow and produce high levels of TNF and iNOS, which are essential in killing bacteria and activating other immune cells [69]. The Ly6C$^{low}$ monocytes are normally characterized by the low expression of CCR2 [68]. They patrol blood vessels to scavenge dead cells, oxidize lipids and pathogens [71]. This thesis compared gene expression between Ly6C$^{low}$ and Ly6C$^{hi}$ monocytes during *L. monocytogenes* infection (Figure 3.2.4). The expression levels of Arg1, TGM2 and Fizz1 in Ly6C$^{low}$ monocytes were greater relative to Ly6C$^{hi}$ monocytes, this suggests an AAM phenotype in the Ly6C$^{low}$ monocytes following infection with *L. monocytogenes* (Figure 3.2.4). The AAM phenotype of Ly6C$^{low}$ monocytes is consistent with these studies as they indicated a wound healing function of Ly6C$^{low}$ monocytes by producing Arg1 and IL-10 [72]. Ly6C$^{low}$ monocytes were gated as CCR2$^{+}$ in this thesis, it is different from the 'classical' Ly6C$^{low}$ monocytes which do not express CCR2 [40]. The high expression of CCR2 in Ly6C$^{low}$ monocytes suggests bone marrow origin. Previous studies have shown that Ly6C$^{hi}$ monocytes are progenitors of Ly6C$^{low}$ monocytes, and decrease expression of Ly6C as their transition [73]. After the resolution of the bacterial infection, Ly6C$^{hi}$ monocytes differentiate into Ly6C$^{low}$ monocytes by downregulating Ly6C expression [40]. It is interesting to find that TNF signalling is required in Ly6C$^{hi}$ monocytes maturation into Ly6C$^{low}$ monocytes (Figure 3.2.2).

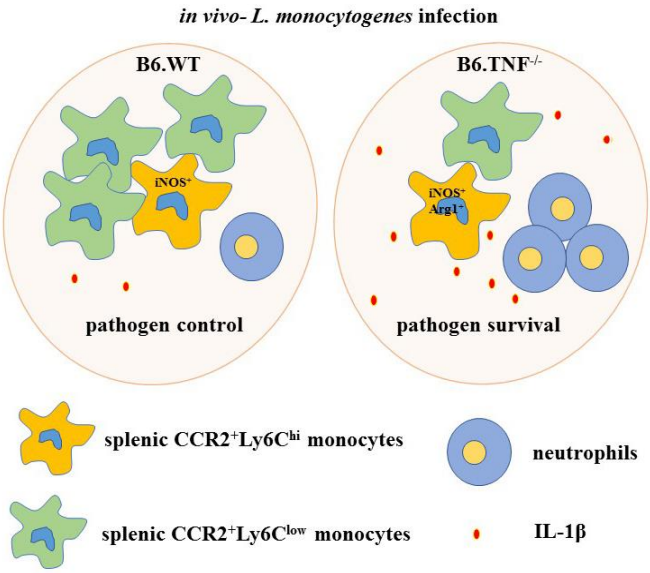


**Figure 6.1 TNF in orchestrate splenic innate immune subsets following the infection of *L. monocytogenes*.** In response to *L. monocytogenes* infection, TNF absence leads to high expression of Arg1 and normal expression of iNOS in CCR2$^{+}$Ly6C$^{hi}$ monocytes. Increased expression of Arg1 results in high bacterial survival and higher number of neutrophils. The release of IL-1β is also enhanced in B6.TNF$^{-/-}$ mice. Draw by XL.

TNF is essential in mediating proper immune responses by maintaining homeostasis during infection. The 'macrophage disappearance reaction', a phenomenon where LPM's migrate from peritoneal cavity to the omentum was apparent in mice after the intraperitoneal inoculation with *L. monocytogenes* (Reviewed in [1]). The reduction of peritoneal macrophage numbers was greater in B6.TNF$^{-/-}$ mice than in wild type controls, which is primarily with the LPM population (Figure 4.2.2). TNF may therefore play a role in maintaining macrophage homeostasis in the context of infection. As LPMs in B6.TNF$^{-/-}$ mice expressed higher levels of CCR2, the reduced numbers of LPMs appears due to the aggressive migration out of the peritoneum cavity. TNF is also required in maintain neutrophil homeostasis and lack of TNF resulted in excessive recruited of neutrophil into infection sites, such as spleen and peritoneum. The bias of AAM differentiation in the lack of TNF results in uncontrolled bacterial infection, hosts have to recruit neutrophils to resolve infection, which may cause tissue damage.

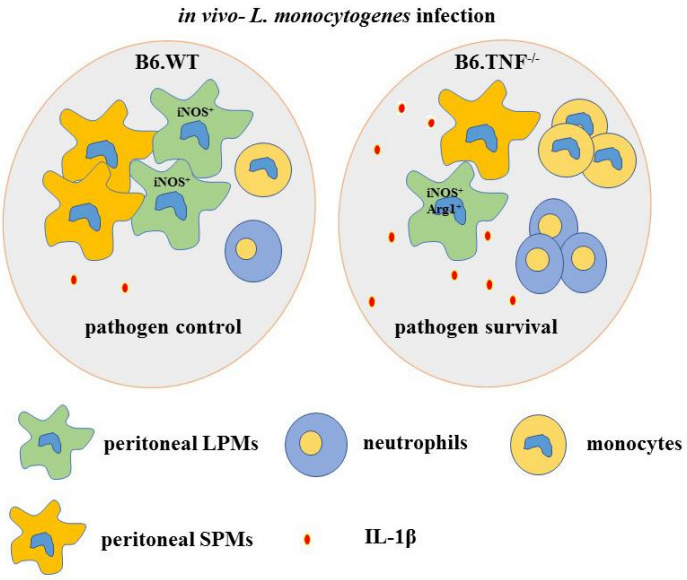


**Figure 6.2 TNF in regulating peritoneal innate immune subsets after *L. monocytogenes* infection.** TNF deficiency results in fewer number of peritoneal macrophages retaining in peritoneum following *L. monocytogenes* infection. LPMs express enhanced level of Arg1 in response to TNF absence. Increased number of neutrophils, monocytes were recruited and increased level of IL-1β was released in B6.TNF$^{-/-}$ mice after *L. monocytogenes* infection. Draw by XL.

### 6.2 Role of TNF in inflammasome activation during *L. monocytogenes* infection

In response to an intracellular stimulus, such as *L. monocytogenes*, macrophage release high levels of IL-1β by the activation of the inflammasome [138]. TNF signalling appears to inhibit macrophage release IL-1β during *L. monocytogenes* infection (Figure 3.2.8). This is controversial to the implication that TNF induces IL-1β production in macrophages [237]. Exogenous TNF triggers macrophage to release more IL-1β via NF-κB activation, whereas the inhibition of NF-κB leads to impaired IL-1β release [237]. The results showed a similar expression of inflammasome elements (caspase-1, pro-caspase-1, NLRP3). The level of pro-IL-1β is upregulated in macrophages from B6.TNF$^{-/-}$ mice. It might result from the higher pro-IL-1β production in the absence of TNF in the stimulation of LPS. Macrophages produce TNF in response to LPS, then TNF stimulates negative feedback in macrophages to restrict the production of IL-1β. The presence of TNF ensures the appropriate production of IL-1β in response to *L. monocytogenes* infection. In the condition of losing TNF, hosts have to release the pro-inflammatory cytokine IL-1β to resolve infection. On the other hand, the high production of IL-1β in the absence of TNF may contribute to the ongoing inflammation during

*L. monocytogenes* infection. The high concentration of IL-1β in sera of patients undergoing anti-TNF therapy might be a diagnose marker for bacterial infections.

### 6.3 Role of TNF in macrophage phagocytosis of tumour cells

Macrophage phagocytosis is a critical function in defence against pathogens. The role of TNF in macrophage phagocytosis was investigated with the tumour cells causing Devil Facial Tumour Disease (DFTD). TNF has no effect on phagocytosis of DFTD cells in the absence of stimulation. Following activation with IFNγ/LPS or LPS (but not IFNγ alone) however, the ability of phagocytosis was enhanced in the absence of TNF (Figure 5.2.4). Although TNF is regarded as a master regulator of inflammatory cytokine release (reviewed in [6]), there are reports that TNF can also inhibit macrophage phagocytic activity. Exogenous TNF has been shown to reduce capacity of macrophages to ingest apoptotic cells [278]. This inhibition appears to be specific for apoptotic cells as it is not observed with beads or antibody-opsonized cells [278]. It is proposed that within the inflammatory environment, the reduced phagocytic ability may 'contribute to the local intensity of the inflammatory response'. Macrophages stimulated with LPS produce TNF which would inhibit macrophage phagocytosis of apoptotic cells in an autocrine manner [265]. TNF is also produced by IFNγ/LPS activated macrophages [94, 267]. IFNγ on its own is unable to activate macrophages to produce TNF, but can do so in the presence of LPS [268]. The situation can be somewhat murky as some studies showed IFNγ primed macrophages can produce TNF [251, 269]. Furthermore, IFNγ appears to restore an impaired ability of macrophages to phagocytose apoptotic cells, which is in a nitric oxide-dependent manner and requires TNF production [251, 269]. However, these conflict results might be due to the macrophages coming from different sources and at different stages of maturation. For example, although TNF has no effect on phagocytosis by immature macrophages, it reduces phagocytosis by mature macrophages [278]. In response to the pro-inflammatory signals of IFNγ/LPS or LPS, the inhibition of TNF in macrophage phagocytosis of tumour cells appears to be 'anti-inflammatory'. This inhibition might be essential in response to pathogens properly to avoid effects of overreaction.

In summary, roles of TNF in macrophage are complex and diverse. Firstly, TNF inhibits the AAM differentiation during *L. monocytogenes* infection, which may leads to the high susceptibility in B6.TNF$^{-/-}$ mice. The high expression of Arg1 in AAMs in B6.TNF$^{-/-}$ mice might lead to impaired elimination of *L. monocytogenes* infection by reducing iNOS activity.

The mechanisms mediated by TNF to inhibit AAM differentiation need to be further elucidated. The inhibition of AAM differentiation of TNF might promote CAM activation in response to bacterial infection. Following infection, the appropriate CAM activation is required in the efficient bacterial elimination. The imbalance of AAM and CAM activation might contribute to the pathology of inflammatory diseases. Secondly, TNF is shown to be an inhibitor of IL-1β release during infection with *L. monocytogenes*. Thirdly, TNF inhibits macrophage phagocytosis of tumour cells after activation with IFNγ/LPS or LPS alone. These findings showed the importance and requirement of TNF in inflammatory responses. As the protective role of TNF in defence against bacterial infection by inhibiting AAM differentiation, bacterial infection should be avoided in the treatment of chronic inflammatory diseases by using anti-TNF antagonists.

# References

## References


1. Cassado, A.d.A., M.R. D'Império Lima, and K.R. Bortoluci, *Revisiting mouse peritoneal macrophages: heterogeneity, development, and function.* Frontiers in immunology, 2015. **6**: p. 225.
2. Biswas, S.K., et al., *Macrophage polarization and plasticity in health and disease.* Immunologic research, 2012. **53**(1-3): p. 11-24.
3. Taylor, P.R., et al., *Pattern recognition receptors and differentiation antigens define murine myeloid cell heterogeneity ex vivo.* European journal of immunology, 2003. **33**(8): p. 2090-2097.
4. Vazquez-Boland, J.A., et al., *Listeria pathogenesis and molecular virulence determinants.* Clin Microbiol Rev, 2001. **14**(3): p. 584-640.
5. Chu, W.M., *Tumor necrosis factor.* Cancer letters, 2013. **328**(2): p. 222-5.
6. Parameswaran, N. and S. Patial, *Tumor necrosis factor-α signaling in macrophages.* Crit Rev Eukaryot Gene Expr, 2010. **20**(2).
7. Buchan, G., et al., *Interleukin-1 and tumour necrosis factor mRNA expression in rheumatoid arthritis: prolonged production of IL-1 alpha.* Clin Exp Immunol, 1988. **73**(3): p. 449.
8. Feldmann, M. and R.N. Maini, *Anti-TNFα therapy of rheumatoid arthritis: what have we learned?* Annual review of immunology, 2001. **19**(1): p. 163-196.
9. Müller, U., et al., *Tumour necrosis factor and lymphotoxin genes map close to H-2D in the mouse major histocompatibility complex.* Nature, 1986. **325**(6101): p. 265-267.
10. Bodmer, J.-L., P. Schneider, and J. Tschopp, *The molecular architecture of the TNF superfamily.* Trends in biochemical sciences, 2002. **27**(1): p. 19-26.
11. TRACEY, K.J. and A. CERAMI, *Tumor necrosis factor: an updated review of its biology.* Critical care medicine, 1993. **21**(10): p. S423-435.
12. Spriggs, D.R., S. Deutsch, and D.W. Kufe, *Genomic structure, induction, and production of TNF-alpha.* Immunology series, 1992. **56**: p. 3-34.
13. Black, R.A., et al., *A metalloproteinase disintegrin that releases tumour necrosis factor-R from cells.* Nature, 1997. **385**(6618): p. 729-733.
14. Wallach, D., *The TNF cytokine family: one track in a road paved by many.* Cytokine, 2013. **63**(3): p. 225-229.
15. Kriegler, M., et al., *A novel form of TNF/cachectin is a cell surface cytotoxic transmembrane protein: ramifications for the complex physiology of TNF.* Cell, 1988. **53**(1): p. 45-53.
16. Deleault, K.M., S.J. Skinner, and S.A. Brooks, *Tristetraprolin regulates TNF TNF-α mRNA stability via a proteasome dependent mechanism involving the combined action of the ERK and p38 pathways.* Molecular immunology, 2008. **45**(1): p. 13-24.
17. Grell, M., et al., *The transmembrane form of tumor necrosis factor is the prime activating ligand of the 80 kDa tumor necrosis factor receptor.* Cell, 1995. **83**(5): p. 793-802.
18. Grell, M., et al., *The type 1 receptor (CD120a) is the high-affinity receptor for soluble tumor necrosis factor.* Proceedings of the National Academy of Sciences, 1998. **95**(2): p. 570-575.
19. Tada, K., et al., *Critical roles of TRAF2 and TRAF5 in tumor necrosis factor-induced NF-κB activation and protection from cell death.* Journal of Biological Chemistry, 2001. **276**(39): p. 36530-36534.
20. Kelliher, M.A., et al., *The death domain kinase RIP mediates the TNF-induced NF-κB signal.* Immunity, 1998. **8**(3): p. 297-303.
21. Devin, A., et al., *The distinct roles of TRAF2 and RIP in IKK activation by TNF-R1: TRAF2 recruits IKK to TNF-R1 while RIP mediates IKK activation.* Immunity, 2000. **12**(4): p. 419-429.
22. Legler, D.F., et al., *Recruitment of TNF receptor 1 to lipid rafts is essential for TNFα-mediated NF-κB activation.* Immunity, 2003. **18**(5): p. 655-664.
23. Pasparakis, M., et al., *Immune and inflammatory responses in TNF alpha-deficient mice: a critical requirement for TNF alpha in the formation of primary B cell follicles, follicular*

*dendritic cell networks and germinal centers, and in the maturation of the humoral immune response.* J Exp Med, 1996. **184**(4): p. 1397-1411.
24. Ichijo, H., et al., *Induction of apoptosis by ASK1, a mammalian MAPKKK that activates SAPK/JNK and p38 signaling pathways.* Science, 1997. **275**(5296): p. 90-94.
25. Liu, X., et al., *Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c.* Cell, 1996. **86**(1): p. 147-57.
26. Van Antwerp, D.J., et al., *Inhibition of TNF-induced apoptosis by NF-κB.* Trends in Cell Biology, 1998. **8**(3): p. 107-111.
27. Wang, Z., et al., *The mitochondrial phosphatase PGAM5 functions at the convergence point of multiple necrotic death pathways.* Cell, 2012. **148**(1-2): p. 228-43.
28. Wang, Z.G., et al., *The Mitochondrial Phosphatase PGAM5 Functions at the Convergence Point of Multiple Necrotic Death Pathways.* Cell, 2012. **148**(1-2): p. 228-243.
29. Witsell, A.L. and L.B. Schook, *Tumor necrosis factor alpha is an autocrine growth regulator during macrophage differentiation.* Proceedings of the National Academy of Sciences, 1992. **89**(10): p. 4754-4758.
30. Vieira, L.Q., et al., *Mice lacking the TNF receptor p55 fail to resolve lesions caused by infection with Leishmania major, but control parasite replication.* J Immunol, 1996. **157**(2): p. 827-35.
31. Rothe, J., et al., *Mice lacking the tumour necrosis factor receptor 1 are resistant to TNF-mediated toxicity but highly susceptible to infection by Listeria monocytogenes.* Nature, 1993. **364**(6440): p. 798-802.
32. Mosser, D.M. and J.P. Edwards, *Exploring the full spectrum of macrophage activation.* Nature reviews immunology, 2008. **8**(12): p. 958-969.
33. Yamamoto, M., et al., *Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway.* Science, 2003. **301**(5633): p. 640-3.
34. Kratochvill, F., et al., *TNF counterbalances the emergence of M2 tumor macrophages.* Cell reports, 2015. **12**(11): p. 1902-1914.
35. Schleicher, U., et al., *TNF-Mediated Restriction of Arginase 1 Expression in Myeloid Cells Triggers Type 2 NO Synthase Activity at the Site of Infection.* Cell reports, 2016. **15**(5): p. 1062-1075.
36. Haldar, M. and K.M. Murphy, *Origin, development, and homeostasis of tissue‐resident macrophages.* Immunological reviews, 2014. **262**(1): p. 25-35.
37. Italiani, P. and D. Boraschi, *From monocytes to M1/M2 macrophages: phenotypical vs. functional differentiation.* M1/M2 Macrophages: The Arginine Fork in the Road to Health and Disease, 2015: p. 47.
38. Wynn, T.A., A. Chawla, and J.W. Pollard, *Macrophage biology in development, homeostasis and disease.* Nature, 2013. **496**(7446): p. 445-455.
39. Ginhoux, F., et al., *Fate mapping analysis reveals that adult microglia derive from primitive macrophages.* Science, 2010. **330**(6005): p. 841-845.
40. Yona, S., et al., *Fate mapping reveals origins and dynamics of monocytes and tissue macrophages under homeostasis.* Immunity, 2013. **38**(1): p. 79-91.
41. Gordon, S. and P.R. Taylor, *Monocyte and macrophage heterogeneity.* Nature Reviews Immunology, 2005. **5**(12): p. 953-964.
42. Mitchell, A.J., B. Roediger, and W. Weninger, *Monocyte homeostasis and the plasticity of inflammatory monocytes.* Cell Immunol, 2014. **291**(1): p. 22-31.
43. Auffray, C., M.H. Sieweke, and F. Geissmann, *Blood monocytes: development, heterogeneity, and relationship with dendritic cells.* Annual review of immunology, 2009. **27**: p. 669-692.
44. Schulz, C., et al., *A lineage of myeloid cells independent of Myb and hematopoietic stem cells.* Science, 2012. **336**(6077): p. 86-90.
45. Bronte, V. and M.J. Pittet, *The spleen in local and systemic regulation of immunity.* Immunity, 2013. **39**(5): p. 806-818.
46. Kurotaki, D., T. Uede, and T. Tamura, *Functions and development of red pulp macrophages.* Microbiology and immunology, 2015. **59**(2): p. 55-62.
47. Davies, L.C., et al., *Tissue-resident macrophages.* Nature immunology, 2013. **14**(10): p. 986-995.

48. Geijtenbeek, T.B., et al., *Marginal zone macrophages express a murine homologue of DC-SIGN that captures blood-borne antigens in vivo.* Blood, 2002. **100**(8): p. 2908-2916.
49. Ghosn, E.E.B., et al., *Two physically, functionally, and developmentally distinct peritoneal macrophage subsets.* Proceedings of the National Academy of Sciences, 2010. **107**(6): p. 2568-2573.
50. Mazzone, A. and G. Ricevuti, *Leukocyte CD11/CD18 integrins: biological and clinical relevance.* Haematologica, 1995. **80**(2): p. 161-175.
51. Taylor, P.R., et al., *Macrophage receptors and immune recognition.* Annu. Rev. Immunol., 2005. **23**: p. 901-944.
52. dos Anjos Cassado, A., et al., *Cellular renewal and improvement of local cell effector activity in peritoneal cavity in response to infectious stimuli.* PLoS One, 2011. **6**(7): p. e22141.
53. Dioszeghy, V., et al., *12/15-Lipoxygenase regulates the inflammatory response to bacterial products in vivo.* The Journal of Immunology, 2008. **181**(9): p. 6514-6524.
54. Barth, M., et al., *Review of the macrophage disappearance reaction.* Journal of leukocyte biology, 1995. **57**(3): p. 361-367.
55. Okabe, Y. and R. Medzhitov, *Tissue-specific signals control reversible program of localization and functional polarization of macrophages.* Cell, 2014. **157**(4): p. 832-844.
56. Davies, L.C., et al., *A quantifiable proliferative burst of tissue macrophages restores homeostatic macrophage populations after acute inflammation.* European journal of immunology, 2011. **41**(8): p. 2155-2164.
57. Jenkins, S.J., et al., *Local macrophage proliferation, rather than recruitment from the blood, is a signature of TH2 inflammation.* Science, 2011. **332**(6035): p. 1284-1288.
58. Wolf, S.A., H.W. Boddeke, and H. Kettenmann, *Microglia in Physiology and Disease.* Annual review of physiology, 2017. **79**: p. 619-643.
59. Michell-Robinson, M.A., et al., *Roles of microglia in brain development, tissue maintenance and repair.* Brain : a journal of neurology, 2015. **138**(Pt 5): p. 1138-59.
60. Dai, X.-M., et al., *Targeted disruption of the mouse colony-stimulating factor 1 receptor gene results in osteopetrosis, mononuclear phagocyte deficiency, increased primitive progenitor cell frequencies, and reproductive defects.* Blood, 2002. **99**(1): p. 111-120.
61. Hume, D.A., et al., *The effect of human recombinant macrophage colony-stimulating factor (CSF-1) on the murine mononuclear phagocyte system in vivo.* The Journal of Immunology, 1988. **141**(10): p. 3405-3409.
62. Wang, Y., et al., *IL-34 is a tissue-restricted ligand of CSF1R required for the development of Langerhans cells and microglia.* Nature immunology, 2012. **13**(8): p. 753-760.
63. Iwasaki, H., et al., *Distinctive and indispensable roles of PU. 1 in maintenance of hematopoietic stem cells and their differentiation.* Blood, 2005. **106**(5): p. 1590-1600.
64. Scott, E.W., et al., *Requirement of transcription factor PU. 1 in the development of multiple hematopoietic lineages.* Science, 1994. **265**(5178): p. 1573-1577.
65. Dahl, R., et al., *Regulation of macrophage and neutrophil cell fates by the PU. 1: C/EBPα ratio and granulocyte colony-stimulating factor.* Nature immunology, 2003. **4**(10): p. 1029-1036.
66. Terry, R.L. and S.D. Miller, *Molecular control of monocyte development.* Cellular immunology, 2014. **291**(1): p. 16-21.
67. Rosas, M., et al., *The transcription factor Gata6 links tissue macrophage phenotype and proliferative renewal.* Science, 2014. **344**(6184): p. 645-648.
68. Geissmann, F., S. Jung, and D.R. Littman, *Blood monocytes consist of two principal subsets with distinct migratory properties.* Immunity, 2003. **19**(1): p. 71-82.
69. Serbina, N.V., et al., *TNF/iNOS-producing dendritic cells mediate innate immune defense against bacterial infection.* Immunity, 2003. **19**(1): p. 59-70.
70. De Trez, C., et al., *iNOS-producing inflammatory dendritic cells constitute the major infected cell type during the chronic Leishmania major infection phase of C57BL/6 resistant mice.* PLoS Pathog, 2009. **5**(6): p. e1000494.
71. Auffray, C., et al., *Monitoring of blood vessels and tissues by a population of monocytes with patrolling behavior.* Science, 2007. **317**(5838): p. 666-670.
72. Shechter, R., et al., *Recruitment of beneficial M2 macrophages to injured spinal cord is orchestrated by remote brain choroid plexus.* Immunity, 2013. **38**(3): p. 555-569.

73. Sunderkötter, C., et al., *Subpopulations of mouse blood monocytes differ in maturation stage and inflammatory response.* The Journal of Immunology, 2004. **172**(7): p. 4410-4417.
74. MacDonald, K.P., et al., *An antibody against the colony-stimulating factor 1 receptor depletes the resident subset of monocytes and tissue-and tumor-associated macrophages but does not inhibit inflammation.* Blood, 2010. **116**(19): p. 3955-3963.
75. Allen, S.J., S.E. Crown, and T.M. Handel, *Chemokine: receptor structure, interactions, and antagonism.* Annu. Rev. Immunol., 2007. **25**: p. 787-820.
76. Serbina, N.V., et al., *Monocyte-mediated defense against microbial pathogens.* Annu Rev Immunol, 2008. **26**: p. 421-52.
77. Peters, W., et al., *Chemokine receptor 2 serves an early and essential role in resistance to Mycobacterium tuberculosis.* Proceedings of the National Academy of Sciences, 2001. **98**(14): p. 7958-7963.
78. De Trez, C., et al., *iNOS-producing inflammatory dendritic cells constitute the major infected cell type during the chronic Leishmania major infection phase of C57BL/6 resistant mice.* PLoS pathogens, 2009. **5**(6): p. e1000494.
79. Jia, T., et al., *Additive roles for MCP-1 and MCP-3 in CCR2-mediated recruitment of inflammatory monocytes during Listeria monocytogenes infection.* The Journal of Immunology, 2008. **180**(10): p. 6846-6853.
80. Proudfoot, A.E., et al., *Glycosaminoglycan binding and oligomerization are essential for the in vivo activity of certain chemokines.* Proceedings of the National Academy of Sciences, 2003. **100**(4): p. 1885-1890.
81. Auffray, C., et al., *CX3CR1+ CD115+ CD135+ common macrophage/DC precursors and the role of CX3CR1 in their response to inflammation.* The Journal of experimental medicine, 2009. **206**(3): p. 595-606.
82. Takeda, K., T. Kaisho, and S. Akira, *Toll-like receptors.* Annual review of immunology, 2003. **21**(1): p. 335-376.
83. Hamilton, T.A., Y. Ohmori, and J. Tebo, *Regulation of chemokine expression by antiinflammatory cytokines.* Immunologic research, 2002. **25**(3): p. 229-245.
84. Kang, S.-J., et al., *Regulation of hierarchical clustering and activation of innate immune cells by dendritic cells.* Immunity, 2008. **29**(5): p. 819-833.
85. Pfeffer, K., et al., *Mice deficient for the 55 kd tumor necrosis factor receptor are resistant to endotoxic shock, yet succumb to L. monocytogenes infection.* Cell, 1993. **73**(3): p. 457-467.
86. Martinez, F.O., et al., *Transcriptional profiling of the human monocyte-to-macrophage differentiation and polarization: new molecules and patterns of gene expression.* The Journal of Immunology, 2006. **177**(10): p. 7303-7311.
87. Hayes, M.P., S.L. Freeman, and R.P. Donnelly, *IFN-γ priming of monocytes enhances LPS-induced TNF production by augmenting both transcription and mRNA stability.* Cytokine, 1995. **7**(5): p. 427-435.
88. Chakravortty, D. and M. Hensel, *Inducible nitric oxide synthase and control of intracellular bacterial pathogens.* Microbes and infection, 2003. **5**(7): p. 621-627.
89. Kobayashi, Y., *The regulatory role of nitric oxide in proinflammatory cytokine expression during the induction and resolution of inflammation.* J Leukoc Biol, 2010. **88**(6): p. 1157-1162.
90. Xie, Q., Y. Kashiwabara, and C. Nathan, *Role of transcription factor NF-kappa B/Rel in induction of nitric oxide synthase.* J Biol Chem, 1994. **269**(7): p. 4705-4708.
91. MacMicking, J., Q.-w. Xie, and C. Nathan, *Nitric oxide and macrophage function.* Annu Rev Immunol, 1997. **15**(1): p. 323-350.
92. Green, S.J., et al., *Nitric oxide: cytokine-regulation of nitric oxide in host resistance to intracellular pathogens.* Immunology letters, 1994. **43**(1-2): p. 87-94.
93. Chan, E.D. and D.W. Riches, *IFN-γ+ LPS induction of iNOS is modulated by ERK, JNK/SAPK, and p38 mapk in a mouse macrophage cell line.* Am J Physiol Lung Cell Mol Physiol, 2001. **280**(3): p. C441-C450.
94. Patel, N.R., et al., *Cell elasticity determines macrophage function.* PloS one, 2012. **7**(9): p. e41024.

95. Ding, A.H., C.F. Nathan, and D.J. Stuehr, *Release of reactive nitrogen intermediates and reactive oxygen intermediates from mouse peritoneal macrophages. Comparison of activating cytokines and evidence for independent production.* J Immunol, 1988. **141**(7): p. 2407-2412.
96. Christopherson, K.S. and D.S. Bredt, *Perspectives series: Nitric oxide and nitric oxide synthases.* J. Clin. Invest, 1997. **100**: p. 2424-2429.
97. Myers, J.T., A.W. Tsang, and J.A. Swanson, *Localized reactive oxygen and nitrogen intermediates inhibit escape of Listeria monocytogenes from vacuoles in activated macrophages.* The Journal of Immunology, 2003. **171**(10): p. 5447-5453.
98. Martinez, F.O., L. Helming, and S. Gordon, *Alternative activation of macrophages: an immunologic functional perspective.* Annu Rev Immunol, 2009. **27**: p. 451-483.
99. Kelly-Welch, A.E., et al., *Interleukin-4 and interleukin-13 signaling connections maps.* Science, 2003. **300**(5625): p. 1527-1528.
100. Martinez, F.O., et al., *Genetic programs expressed in resting and IL-4 alternatively activated mouse and human macrophages: similarities and differences.* Blood, 2013. **121**(9): p. e57-e69.
101. Astarie-Dequeker, C., et al., *The mannose receptor mediates uptake of pathogenic and nonpathogenic mycobacteria and bypasses bactericidal responses in human macrophages.* Infection and immunity, 1999. **67**(2): p. 469-477.
102. Montaner, L.J., et al., *Type 1 and type 2 cytokine regulation of macrophage endocytosis: differential activation by IL-4/IL-13 as opposed to IFN-γ or IL-10.* The Journal of Immunology, 1999. **162**(8): p. 4606-4613.
103. Nair, M.G., et al., *Alternatively activated macrophage-derived RELM-α is a negative regulator of type 2 inflammation in the lung.* Journal of Experimental Medicine, 2009. **206**(4): p. 937-952.
104. Rutschman, R., et al., *Cutting edge: Stat6-dependent substrate depletion regulates nitric oxide production.* The Journal of Immunology, 2001. **166**(4): p. 2173-2177.
105. El Kasmi, K.C., et al., *Toll-like receptor–induced arginase 1 in macrophages thwarts effective immunity against intracellular pathogens.* Nature immunology, 2008. **9**(12): p. 1399-1406.
106. Benoit, M., B. Desnues, and J.-L. Mege, *Macrophage polarization in bacterial infections.* The Journal of Immunology, 2008. **181**(6): p. 3733-3739.
107. Van Dyken, S.J. and R.M. Locksley, *Interleukin-4-and interleukin-13-mediated alternatively activated macrophages: roles in homeostasis and disease.* Annual review of immunology, 2013. **31**: p. 317-343.
108. Pesce, J.T., et al., *Arginase-1–expressing macrophages suppress Th2 cytokine–driven inflammation and fibrosis.* PLoS Pathog, 2009. **5**(4): p. e1000371.
109. Albina, J.E., et al., *Temporal expression of different pathways of 1-arginine metabolism in healing wounds.* The Journal of Immunology, 1990. **144**(10): p. 3877-3880.
110. Gordon, S. and F.O. Martinez, *Alternative activation of macrophages: mechanism and functions.* Immunity, 2010. **32**(5): p. 593-604.
111. Martinez, F.O. and S. Gordon, *The M1 and M2 paradigm of macrophage activation: time for reassessment.* F1000Prime Rep, 2014. **6**(13.10): p. 12703.
112. Wilson, E.H., et al., *A critical role for IL-10 in limiting inflammation during toxoplasmic encephalitis.* Journal of neuroimmunology, 2005. **165**(1): p. 63-74.
113. Li, C., et al., *Pathology of Plasmodium chabaudi chabaudi infection and mortality in interleukin-10-deficient mice are ameliorated by anti-tumor necrosis factor alpha and exacerbated by anti-transforming growth factor β antibodies.* Infection and immunity, 2003. **71**(9): p. 4850-4856.
114. Demangel, C., P. Bertolino, and W.J. Britton, *Autocrine IL‐10 impairs dendritic cell (DC)‐derived immune responses to mycobacterial infection by suppressing DC trafficking to draining lymph nodes and local IL‐12 production.* European journal of immunology, 2002. **32**(4): p. 994-1002.
115. Joss, A., et al., *IL-10 directly acts on T cells by specifically altering the CD28 co-stimulation pathway.* European journal of immunology, 2000. **30**(6): p. 1683-1690.
116. Song, E., et al., *Influence of alternatively and classically activated macrophages on fibrogenic activities of human fibroblasts.* Cellular immunology, 2000. **204**(1): p. 19-28.

117. Bingle, L., N. Brown, and C. Lewis, *The role of tumour‐associated macrophages in tumour progression: implications for new anticancer therapies.* The Journal of pathology, 2002. **196**(3): p. 254-265.
118. Balkwill, F.R. and A. Mantovani. *Cancer-related inflammation: common themes and therapeutic opportunities*. in *Seminars in cancer biology*. 2012. Elsevier.
119. Stahl, P.D. and R.A.B. Ezekowitz, *The mannose receptor is a pattern recognition receptor involved in host defense.* Current opinion in immunology, 1998. **10**(1): p. 50-55.
120. Lewis, C.E. and J.W. Pollard, *Distinct role of macrophages in different tumor microenvironments.* Cancer research, 2006. **66**(2): p. 605-612.
121. Chan, K.S., et al., *Identification, molecular characterization, clinical prognosis, and therapeutic targeting of human bladder tumor-initiating cells.* Proceedings of the National Academy of Sciences of the United States of America, 2009. **106**(33): p. 14016-14021.
122. Aderem, A. and D.M. Underhill, *Mechanisms of phagocytosis in macrophages.* Annu Rev Immunol, 1999. **17**(1): p. 593-623.
123. Aderem, A., *How to eat something bigger than your head.* Cell, 2002. **110**(1): p. 5-8.
124. Erwig, L.-P. and P.M. Henson, *Immunological consequences of apoptotic cell phagocytosis.* The American journal of pathology, 2007. **171**(1): p. 2-8.
125. Vieira, O., R. Botelho, and S. Grinstein, *Phagosome maturation: aging gracefully.* Biochem. J, 2002. **366**: p. 689-704.
126. Fraser, I.P., H. Koziel, and R.A.B. Ezekowitz. *The serum mannose-binding protein and the macrophage mannose receptor are pattern recognition molecules that link innate and adaptive immunity*. in *Seminars in immunology*. 1998. Elsevier.
127. Stein, M. and S. Gordon, *Regulation of tumor necrosis factor (TNF) release by murine peritoneal macrophages: role of cell stimulation and specific phagocytic plasma membrane receptors.* European journal of immunology, 1991. **21**(2): p. 431-437.
128. Yamamoto, Y., T.W. Klein, and H. Friedman, *Involvement of mannose receptor in cytokine interleukin-1beta (IL-1beta), IL-6, and granulocyte-macrophage colony-stimulating factor responses, but not in chemokine macrophage inflammatory protein 1beta (MIP-1beta), MIP-2, and KC responses, caused by attachment of Candida albicans to macrophages.* Infection and immunity, 1997. **65**(3): p. 1077-1082.
129. Carroll, M.C., *The role of complement and complement receptors in induction and regulation of immunity.* Annual review of immunology, 1998. **16**(1): p. 545-568.
130. Wright, S.D. and S.C. Silverstein, *Receptors for C3b and C3bi promote phagocytosis but not the release of toxic oxygen from human phagocytes.* J Exp Med, 1983. **158**(6): p. 2016-2023.
131. Joshi, T., J.P. Butchar, and S. Tridandapani, *Fcγ receptor signaling in phagocytes.* Int J Hematol, 2006. **84**(3): p. 210-216.
132. Crowley, M.T., et al., *A critical role for Syk in signal transduction and phagocytosis mediated by Fcγ receptors on macrophages.* J Exp Med, 1997. **186**(7): p. 1027-1039.
133. Araki, N., M.T. Johnson, and J.A. Swanson, *A role for phosphoinositide 3-kinase in the completion of macropinocytosis and phagocytosis by macrophages.* Journal of Cell Biology, 1996. **135**(5): p. 1249-1260.
134. Ridley, A.J., et al., *The small GTP-binding protein rac regulates growth factor-induced membrane ruffling.* Cell, 1992. **70**(3): p. 401-410.
135. Marshansky, V. and M. Futai, *The V-type H+-ATPase in vesicular trafficking: targeting, regulation and function.* Current opinion in cell biology, 2008. **20**(4): p. 415-426.
136. Duclos, S., et al., *Rab5 regulates the kiss and run fusion between phagosomes and endosomes and the acquisition of phagosome leishmanicidal properties in RAW 264.7 macrophages.* Journal of cell science, 2000. **113**(19): p. 3531-3541.
137. Harrison, R.E., et al., *Phagosomes fuse with late endosomes and/or lysosomes by extension of membrane protrusions along microtubules: role of Rab7 and RILP*. Molecular and cellular biology, 2003. **23**(18): p. 6494-6506.
138. Franchi, L., R. Muñoz-Planillo, and G. Núñez, *Sensing and reacting to microbes through the inflammasomes.* Nature immunology, 2012. **13**(4): p. 325-332.
139. Meylan, E., J. Tschopp, and M. Karin, *Intracellular pattern recognition receptors in the host response.* Nature, 2006. **442**(7098): p. 39-44.

140. Takeda, K. and S. Akira, *Toll-like receptors in innate immunity.* Int Immunol, 2005. **17**(1): p. 1-14.
141. Franchi, L., et al., *Function of Nod ‐like receptors in microbial recognition and host defense.* Immunological reviews, 2009. **227**(1): p. 106-128.
142. Menu, P. and J. Vince, *The NLRP3 inflammasome in health and disease: the good, the bad and the ugly.* Clinical & Experimental Immunology, 2011. **166**(1): p. 1-15.
143. Kanneganti, T.-D., et al., *Bacterial RNA and small antiviral compounds activate caspase-1 through cryopyrin/Nalp3.* Nature, 2006. **440**(7081): p. 233-236.
144. Bauernfeind, F.G., et al., *Cutting edge: NF-κB activating pattern recognition and cytokine receptors license NLRP3 inflammasome activation by regulating NLRP3 expression.* The Journal of Immunology, 2009. **183**(2): p. 787-791.
145. Li, P., et al., *Mice deficient in IL-1β-converting enzyme are defective in production of mature IL-1β and resistant to endotoxic shock.* Cell, 1995. **80**(3): p. 401-411.
146. Fink, S.L. and B.T. Cookson, *Apoptosis, pyroptosis, and necrosis: mechanistic description of dead and dying eukaryotic cells.* Infection and immunity, 2005. **73**(4): p. 1907-1916.
147. Petrilli, V., et al., *Activation of the NALP3 inflammasome is triggered by low intracellular potassium concentration.* Cell Death & Differentiation, 2007. **14**(9): p. 1583-1589.
148. Ferrari, D., et al., *The P2X7 receptor: a key player in IL-1 processing and release.* The Journal of Immunology, 2006. **176**(7): p. 3877-3883.
149. Dostert, C., et al., *Innate immune activation through Nalp3 inflammasome sensing of asbestos and silica.* Science, 2008. **320**(5876): p. 674-677.
150. Nakahira, K., et al., *Autophagy proteins regulate innate immune responses by inhibiting the release of mitochondrial DNA mediated by the NALP3 inflammasome.* Nature immunology, 2011. **12**(3): p. 222-230.
151. Ting, J.P.Y., S.B. Willingham, and D.T. Bergstralh, *NLRs at the intersection of cell death and immunity.* Nature Reviews Immunology, 2008. **8**(5): p. 372-379.
152. Martinon, F. and J. Tschopp, *Inflammatory caspases and inflammasomes: master switches of inflammation.* Cell death and differentiation, 2007. **14**(1): p. 10-22.
153. Bryant, C. and K.A. Fitzgerald, *Molecular mechanisms involved in inflammasome activation.* Trends Cell Biol, 2009. **19**(9): p. 455-64.
154. Broz, P., et al., *Redundant roles for inflammasome receptors NLRP3 and NLRC4 in host defense against Salmonella.* J Exp Med, 2010. **207**(8): p. 1745-55.
155. Lightfield, K.L., et al., *Critical function for Naip5 in inflammasome activation by a conserved carboxy-terminal domain of flagellin.* Nature Immunology, 2008. **9**(10): p. 1171-1178.
156. Suzuki, T., et al., *Differential regulation of caspase-1 activation, pyroptosis, and autophagy via Ipaf and ASC in Shigella-infected macrophages.* Plos Pathogens, 2007. **3**(8): p. 1082-1091.
157. Master, S.S., et al., *Mycobacterium tuberculosis prevents inflammasome activation.* Cell Host Microbe, 2008. **3**(4): p. 224-232.
158. Warren, S.E., et al., *Cutting edge: Cytosolic bacterial DNA activates the inflammasome via Aim2.* The Journal of Immunology, 2010. **185**(2): p. 818-821.
159. Rathinam, V.A., et al., *The AIM2 inflammasome is essential for host defense against cytosolic bacteria and DNA viruses.* Nature immunology, 2010. **11**(5): p. 395-402.
160. Cai, X., Y.H. Chiu, and Z.J. Chen, *The cGAS-cGAMP-STING pathway of cytosolic DNA sensing and signaling.* Mol Cell, 2014. **54**(2): p. 289-96.
161. Xiao, T.S. and K.A. Fitzgerald, *The cGAS-STING pathway for DNA sensing.* Mol Cell, 2013. **51**(2): p. 135-9.
162. Martinon, F., et al., *Gout-associated uric acid crystals activate the NALP3 inflammasome.* Nature, 2006. **440**(7081): p. 237-241.
163. Maedler, K., et al., *Interleukin-1 beta targeted therapy for type 2 diabetes.* Expert Opin Biol Th, 2009. **9**(9): p. 1177-1188.
164. Zhou, R.B., et al., *Thioredoxin-interacting protein links oxidative stress to inflammasome activation.* Nature Immunology, 2010. **11**(2): p. 136-U51.

165. Gattorno, M., et al., *Pattern of interleukin‐1β secretion in response to lipopolysaccharide and ATP before and after interleukin‐1 blockade in patients with CIAS1 mutations.* Arthritis & Rheumatism, 2007. **56**(9): p. 3138-3148.
166. Hawkins, P.N., H.J. Lachmann, and M.F. McDermott, *Interleukin-1–receptor antagonist in the Muckle–Wells syndrome.* New England Journal of Medicine, 2003. **348**(25): p. 2583-2584.
167. Kalliolias, G.D. and S.N.C. Liossis, *The future of the IL-1 receptor antagonist anakinra: from rheumatoid arthritis to adult-onset Still's disease and systemic-onset juvenile idiopathic arthritis.* Expert Opin Inv Drug, 2008. **17**(3): p. 349-359.
168. Murray, E.G.D., R.A. Webb, and M.B.R. Swann, *A disease of rabbits characterised by a large mononuclear leucocytosis, caused by a hitherto undescribed bacillus Bacterium monocytogenes (n.sp.).* The Journal of Pathology and Bacteriology, 1926. **29**(4): p. 407-439.
169. Schuchat, A., B. Swaminathan, and C.V. Broome, *Epidemiology of human listeriosis.* Clinical Microbiology Reviews, 1991. **4**(2): p. 169-183.
170. Gellin, B. and C. Broome, *Listeriosis.* JAMA: the journal of the American Medical Association, 1989. **261**(9): p. 1313-1320.
171. Gaillard, J.-L., et al., *In vitro model of penetration and intracellular growth of Listeria monocytogenes in the human enterocyte-like cell line Caco-2.* Infection and immunity, 1987. **55**(11): p. 2822-2829.
172. Kuhn, M., S. Kathariou, and W. Goebel, *Hemolysin supports survival but not entry of the intracellular bacterium Listeria monocytogenes.* Infection and immunity, 1988. **56**(1): p. 79-82.
173. Zenewicz, L.A. and H. Shen, *Innate and adaptive immune responses to< i> Listeria monocytogenes</i>: a short overview.* Microbes and Infection, 2007. **9**(10): p. 1208-1215.
174. Dussurget, O., J. Pizarro-Cerda, and P. Cossart, *Molecular determinants of Listeria monocytogenes virulence.* Annu. Rev. Microbiol., 2004. **58**: p. 587-610.
175. Alouf, J.E., S.J. Billington, and B.H. Jost, *Repertoire and general features of the family of cholesterol-dependent cytolysins.* The comprehensive sourcebook of bacterial protein toxins, 2006. **3**.
176. Geoffroy, C., et al., *Purification, characterization, and toxicity of the sulfhydryl-activated hemolysin listeriolysin O from Listeria monocytogenes.* Infection and Immunity, 1987. **55**(7): p. 1641-1646.
177. Henry, R., et al., *Cytolysin‐dependent delay of vacuole maturation in macrophages infected with Listeria monocytogenes.* Cell Microbiol, 2006. **8**(1): p. 107-119.
178. Pistor, S., et al., *The ActA protein of Listeria monocytogenes acts as a nucleator inducing reorganization of the actin cytoskeleton.* The EMBO Journal, 1994. **13**(4): p. 758.
179. Kocks, C., et al., *L. monocytogenes-induced actin assembly requires the actA gene product, a surface protein.* Cell, 1992. **68**(3): p. 521-31.
180. Lasa, I., et al., *The amino-terminal part of ActA is critical for the actin-based motility of Listeria monocytogenes; the central proline-rich region acts as a stimulator.* Molecular Microbiology, 1995. **18**(3): p. 425-436.
181. Lasa, I., et al., *Identification of two regions in the N-terminal domain of ActA involved in the actin comet tail formation by Listeria monocytogenes.* EMBO J, 1997. **16**(7): p. 1531-40.
182. Serbina, N.V. and E.G. Pamer, *Monocyte emigration from bone marrow during bacterial infection requires signals mediated by chemokine receptor CCR2.* Nature immunology, 2006. **7**(3): p. 311-317.
183. Serbina, N.V., et al., *Sequential MyD88-independent and -dependent activation of innate immune responses to intracellular bacterial infection.* Immunity, 2003. **19**(6): p. 891-901.
184. Seki, E., et al., *Critical roles of myeloid differentiation factor 88-dependent proinflammatory cytokine release in early phase clearance of Listeria monocytogenes in mice.* The Journal of Immunology, 2002. **169**(7): p. 3863-3868.
185. MacMicking, J.D., et al., *Altered responses to bacterial infection and endotoxic shock in mice lacking inducible nitric oxide synthase.* Cell, 1995. **81**(4): p. 641-650.

186. Cole, C., et al., *Nitric Oxide Increases Susceptibility of Toll-like Receptor-Activated Macrophages to Spreading< i> Listeria monocytogenes</i>*. Immunity, 2012. **36**(5): p. 807-820.
187. Williams, M.A., R.L. Schmidt, and L.L. Lenz, *Early events regulating immunity and pathogenesis during Listeria monocytogenes infection.* Trends in immunology, 2012. **33**(10): p. 488-495.
188. Mariathasan, S., et al., *Cryopyrin activates the inflammasome in response to toxins and ATP.* Nature, 2006. **440**(7081): p. 228-232.
189. Hara, H., et al., *Dependency of caspase-1 activation induced in macrophages by Listeria monocytogenes on cytolysin, listeriolysin O, after evasion from phagosome into the cytoplasm.* The Journal of Immunology, 2008. **180**(12): p. 7859-7868.
190. Franchi, L., et al., *Cytosolic flagellin requires Ipaf for activation of caspase-1 and interleukin 1β in salmonella-infected macrophages.* Nature immunology, 2006. **7**(6): p. 576-582.
191. Hawkins, C., et al., *Emerging disease and population decline of an island endemic, the Tasmanian devil Sarcophilus harrisii.* Biol Conserv, 2006. **131**(2): p. 307-324.
192. Woods, G.M., et al., *Immunology of a transmissible cancer spreading among Tasmanian devils.* The Journal of Immunology, 2015. **195**(1): p. 23-29.
193. Hollings, T., et al., *Trophic Cascades Following the Disease-Induced Decline of an Apex Predator, the Tasmanian Devil.* Conservation Biology, 2014. **28**(1): p. 63-75.
194. Pemberton, D. and D. Renouf, *A field-study of communication and social-behavior of the Tasmanian Devil at feeding sites.* Australian journal of zoology, 1993. **41**(5): p. 507-526.
195. Hamede, R.K., H. McCallum, and M. Jones, *Seasonal, demographic and density‐related patterns of contact between Tasmanian devils (Sarcophilus harrisii): Implications for transmission of devil facial tumour disease.* Austral Ecology, 2008. **33**(5): p. 614-622.
196. Pye, R., G. Woods, and A. Kreiss, *Devil Facial Tumor Disease.* Veterinary pathology, 2015: p. 0300985815616444.
197. Hemsley, S., P. Canfield, and A. Husband, *Immunohistological staining of lymphoid tissue in four Australian marsupial species using species cross-reactive antibodies.* Immunology & Cell Biology, 1995. **73**(4).
198. Murchison, E.P., et al., *The Tasmanian devil transcriptome reveals Schwann cell origins of a clonally transmissible cancer.* Science, 2010. **327**(5961): p. 84-87.
199. Siddle, H.V., et al., *Reversible epigenetic down-regulation of MHC molecules by devil facial tumour disease illustrates immune escape by a contagious cancer.* PNAS, 2013. **110**(13): p. 5103-5108.
200. Pye, R., et al., *Demonstration of immune responses against devil facial tumour disease in wild Tasmanian devils.* Biology letters, 2016. **12**(10).
201. Kreiss, A., et al., *Evidence for induction of humoral and cytotoxic immune responses against devil facial tumor disease cells in Tasmanian devils (Sarcophilus harrisii) immunized with killed cell preparations.* Vaccine, 2015. **33**(26): p. 3016-3025.
202. Kreiss, A., et al., *A histological and immunohistochemical analysis of lymphoid tissues of the Tasmanian devil.* Anat Rec, 2009. **292**(5): p. 611-620.
203. Kreiss, A., et al., *Assessment of cellular immune responses of healthy and diseased Tasmanian devils (Sarcophilus harrisii).* Developmental & Comparative Immunology, 2008. **32**(5): p. 544-553.
204. Brown, G.K., et al., *Natural killer cell mediated cytotoxic responses in the Tasmanian devil.* PLoS One, 2011. **6**(9): p. e24475-e24475.
205. Kreiss, A., B. Wells, and G.M. Woods, *The humoral immune response of the Tasmanian devil (Sarcophilus harrisii) against horse red blood cells.* Veterinary immunology and immunopathology, 2009. **130**(1): p. 135-137.
206. O'brien, S., et al., *Genetic basis for species vulnerability in the cheetah.* Science, 1985. **227**(4693): p. 1428-1434.
207. McGuire, K.L., W.R. Duncan, and P.W. Tucker, *Syrian hamster DNA shows limited polymorphism at class I-like loci.* Immunogenetics, 1985. **22**(3): p. 257-268.

208. Miller, W., et al., *Genetic diversity and population structure of the endangered marsupial Sarcophilus harrisii (Tasmanian devil).* Proceedings of the National Academy of Sciences, 2011. **108**(30): p. 12348-12353.
209. Kreiss, A., et al., *Allorecognition in the Tasmanian devil (Sarcophilus harrisii), an endangered marsupial species with limited genetic diversity.* PLoS one, 2011. **6**(7): p. e22402-e22402.
210. Chang, C.-C., M. Campoli, and S. Ferrone, *Classical and nonclassical HLA class I antigen and NK cell–activating ligand changes in malignant cells: current challenges and future directions.* Advances in cancer research, 2005. **93**: p. 189-234.
211. Brown, G.K., et al., *Natural killer cell mediated cytotoxic responses in the Tasmanian devil.* PLoS One, 2011. **6**(9): p. e24475.
212. Letterio, J.J. and A.B. Roberts, *Regulation of immune responses by TGF-β*.* Annual review of immunology, 1998. **16**(1): p. 137-161.
213. Saraiva, M. and A. O'Garra, *The regulation of IL-10 production by immune cells.* Nature Reviews Immunology, 2010. **10**(3): p. 170-181.
214. Morris, K. and K. Belov, *Does the devil facial tumour produce immunosuppressive cytokines as an immune evasion strategy?* Veterinary immunology and immunopathology, 2013. **153**(1): p. 159-164.
215. A, K., *The Immune Responses of the Tasmanian Devil (Sarcophilus harrisii) and the Devil Facial Tumour Disease*, in *Hobart, Tasmania, Australia* 2009, University of Tasmania.
216. Tansey, M.G. and D.E. Szymkowski, *The TNF superfamily in 2009: new pathways, new indications, and new drugs.* Drug discovery today, 2009. **14**(23-24): p. 1082-8.
217. Smith, L.S., M. Nelson, and C.R. Dolder, *Certolizumab pegol: a TNF-{alpha} antagonist for the treatment of moderate-to-severe Crohn's disease.* The Annals of pharmacotherapy, 2010. **44**(2): p. 333-42.
218. Raychaudhuri, S.P., et al., *Incidence and nature of infectious disease in patients treated with anti-TNF agents.* Autoimmunity reviews, 2009. **9**(2): p. 67-81.
219. De Leonardis, F., et al., *Visceral leishmaniasis and anti-TNF-alpha therapy: case report and review of the literature.* Clinical and experimental rheumatology, 2009. **27**(3): p. 503-6.
220. Körner, H., et al., *Distinct roles for lymphotoxin-and tumour necrosis factor in lymphoid tissue organogenesis and spatial organisation defined in gene targeted C57BL/6 mice.* Eur. J. Immunol, 1997. **27**: p. 2600-2609.
221. Mo, J. and J.A. Duncan, *The Inflammasome: Methods and Protocols*, in *Assessing ATP binding and hydrolysis by NLR proteins*. 2013.
222. Pearse, A.-M., et al., *Evolution in a transmissible cancer: a study of the chromosomal changes in devil facial tumor (DFT) as it spreads through the wild Tasmanian devil population.* Cancer Genet, 2012. **205**(3): p. 101-112.
223. Wilhelm, P., et al., *Rapidly fatal leishmaniasis in resistant C57BL/6 mice lacking TNF*. J Immunol, 2001. **166**(6): p. 4012-4019.
224. Segal, A.W., *How neutrophils kill microbes.* Annual review of immunology, 2005. **23**: p. 197.
225. Czuprynski, C., et al., *Administration of anti-granulocyte mAb RB6-8C5 impairs the resistance of mice to Listeria monocytogenes infection.* The Journal of Immunology, 1994. **152**(4): p. 1836-1846.
226. Boxio, R., et al., *Mouse bone marrow contains large numbers of functionally competent neutrophils.* Journal of leukocyte biology, 2004. **75**(4): p. 604-611.
227. Nahrendorf, M., et al., *The healing myocardium sequentially mobilizes two monocyte subsets with divergent and complementary functions.* The Journal of experimental medicine, 2007. **204**(12): p. 3037-3047.
228. Varol, C., et al., *Monocytes give rise to mucosal, but not splenic, conventional dendritic cells.* The Journal of experimental medicine, 2007. **204**(1): p. 171-180.
229. Crane, M.J., et al., *The monocyte to macrophage transition in the murine sterile wound.* PloS one, 2014. **9**(1): p. e86660.
230. Tessier, P.A., et al., *Chemokine networks in vivo: involvement of CXC and CC chemokines in neutrophil extravasation in vivo in response to TNF-alpha.* The Journal of Immunology, 1997. **159**(7): p. 3595-3602.

231. Kobayashi, Y., *The role of chemokines in neutrophil biology.* Frontiers in bioscience: a journal and virtual library, 2007. **13**: p. 2400-2407.
232. Musicki, K., et al., *Differential requirements for soluble and transmembrane tumor necrosis factor in the immunological control of primary and secondary Listeria monocytogenes infection.* Infection and immunity, 2006. **74**(6): p. 3180-3189.
233. Witter, A.R., B.M. Okunnu, and R.E. Berg, *The Essential Role of Neutrophils during Infection with the Intracellular Bacterial Pathogen Listeria monocytogenes.* The Journal of Immunology, 2016. **197**(5): p. 1557-1565.
234. Liu, M., et al., *Formylpeptide receptors are critical for rapid neutrophil mobilization in host defense against Listeria monocytogenes.* Scientific reports, 2012. **2**: p. 786.
235. Noubade, R., et al., *NRROS negatively regulates reactive oxygen species during host defence and autoimmunity.* Nature, 2014. **509**(7499): p. 235-239.
236. Carlin, L.M., et al., *Nr4a1-dependent Ly6C low monocytes monitor endothelial cells and orchestrate their disposal.* Cell, 2013. **153**(2): p. 362-375.
237. Álvarez, S. and M.Á. Muñoz-Fernández, *TNF-A may mediate inflammasome activation in the absence of bacterial infection in more than one way.* PloS one, 2013. **8**(8): p. e71477.
238. Vázquez-Boland, J.A., et al., *Listeria pathogenesis and molecular virulence determinants.* Clinical microbiology reviews, 2001. **14**(3): p. 584-640.
239. Bronte, V. and P. Zanovello, *Regulation of immune responses by L-arginine metabolism.* Nature Reviews Immunology, 2005. **5**(8): p. 641-654.
240. Cain, D.W., et al., *Identification of a tissue-specific, C/EBPβ-dependent pathway of differentiation for murine peritoneal macrophages.* The Journal of Immunology, 2013. **191**(9): p. 4665-4675.
241. Wang, J. and P. Kubes, *A reservoir of mature cavity macrophages that can rapidly invade visceral organs to affect tissue repair.* Cell, 2016. **165**(3): p. 668-678.
242. Rogers, H.W. and E.R. Unanue, *Neutrophils are involved in acute, nonspecific resistance to Listeria monocytogenes in mice.* Infection and immunity, 1993. **61**(12): p. 5090-5096.
243. Weber, C., et al., *Downregulation by tumor necrosis factor-α of monocyte CCR2 expression and monocyte chemotactic protein-1-induced transendothelial migration is antagonized by oxidized low-density lipoprotein: A potential mechanism of monocyte retention in atherosclerotic lesions.* Atherosclerosis, 1999. **145**(1): p. 115-123.
244. Ruffell, D., et al., *A CREB-C/EBPbeta cascade induces M2 macrophage-specific gene expression and promotes muscle injury repair.* Proc Natl Acad Sci U S A, 2009. **106**(41): p. 17475-80.
245. Lawrence, T. and G. Natoli, *Transcriptional regulation of macrophage polarization: enabling diversity with identity.* Nature reviews. Immunology, 2011. **11**(11): p. 750-61.
246. Scholzen, T. and J. Gerdes, *The Ki-67 protein: from the known and the unknown.* Journal of cellular physiology, 2000. **182**(3): p. 311-22.
247. Bleriot, C., et al., *Liver-resident macrophage necroptosis orchestrates type 1 microbicidal inflammation and type-2-mediated tissue repair during bacterial infection.* Immunity, 2015. **42**(1): p. 145-58.
248. Underhill, D.M. and A. Ozinsky, *Phagocytosis of microbes: complexity in action.* Annu Rev Immunol, 2002. **20**(1): p. 825-852.
249. Borges, V.M., et al., *TNFα inhibits apoptotic cell clearance in the lung, exacerbating acute inflammation.* Am J Physiol Lung Cell Mol Physiol, 2009. **297**(4): p. L586-L595.
250. Leichtle, A., et al., *CC chemokine ligand 3 overcomes the bacteriocidal and phagocytic defect of macrophages and hastens recovery from experimental otitis media in TNF-/- mice.* J Immunol, 2010. **184**(6): p. 3087-97.
251. Fernandez-Boyanapalli, R., et al., *Impaired phagocytosis of apoptotic cells by macrophages in chronic granulomatous disease is reversed by IFN-γ in a nitric oxide-dependent manner.* J Immunol, 2010. **185**(7): p. 4030-4041.
252. Portnoy, D.A., V. Auerbuch, and I.J. Glomski, *The cell biology of Listeria monocytogenes infection.* J Cell Biol, 2002. **158**(3): p. 409-414.
253. Birmingham, C.L., et al., *Listeriolysin O allows Listeria monocytogenes replication in macrophage vacuoles.* Nature, 2008. **451**(7176): p. 350-354.

254. Kocks, C., et al., *L. monocytogenes-induced actin assembly requires the actA gene product, a surface protein.* Cell, 1992. **68**(3): p. 521-531.
255. Pyecroft, S.B., et al., *Towards a case definition for devil facial tumour disease: what is it?* EcoHealth, 2007. **4**(3): p. 346-351.
256. Tjelle, T.E., T. Løvdal, and T. Berg, *Phagosome dynamics and function.* Bioessays, 2000. **22**(3): p. 255-263.
257. Higginbotham, J.N., T. Lin, and S. Pruett, *Effect of macrophage activation on killing of Listeria monocytogenes. Roles of reactive oxygen or nitrogen intermediates, rate of phagocytosis, and retention of bacteria in endosomes.* Clinical & Experimental Immunology, 1992. **88**(3): p. 492-498.
258. Gonçalves, R. and D.M. Mosser, *The isolation and characterization of murine macrophages.* Current protocols in immunology, 2008: p. 14.1. 1-14.1. 16.
259. Leijh, P., et al., *Effect of thioglycolate on phagocytic and microbicidal activities of peritoneal macrophages.* Infection and immunity, 1984. **46**(2): p. 448-452.
260. Kreiss, A., et al., *Evidence for induction of humoral and cytotoxic immune responses against devil facial tumor disease cells in Tasmanian devils (Sarcophilus harrisii) immunized with killed cell preparations.* Vaccine, 2015. **33**(26): p. 3016-3025.
261. Tovar, C., et al., *Regression of devil facial tumour disease following immunotherapy in immunised Tasmanian devils.* Sci Rep, 2017. **7**.
262. Alvey, C. and D.E. Discher, *Engineering macrophages to eat cancer: from "marker of self" CD47 and phagocytosis to differentiation.* J Leukoc Biol, 2017.
263. Flies, A.S., et al., *Comparative Analysis of Immune Checkpoint Molecules and Their Potential Role in the Transmissible Tasmanian Devil Facial Tumor Disease.* Front Immunol, 2017. **8**: p. 513.
264. McPhillips, K., et al., *TNF-α inhibits macrophage clearance of apoptotic cells via cytosolic phospholipase A2 and oxidant-dependent mechanisms.* J Immunol, 2007. **178**(12): p. 8117-8126.
265. Feng, X., et al., *Lipopolysaccharide inhibits macrophage phagocytosis of apoptotic neutrophils by regulating the production of tumour necrosis factor α and growth arrest‐specific gene 6.* Immunology, 2011. **132**(2): p. 287-295.
266. Michlewska, S., et al., *Macrophage phagocytosis of apoptotic neutrophils is critically regulated by the opposing actions of pro-inflammatory and anti-inflammatory agents: key role for TNF-α.* The FASEB Journal, 2009. **23**(3): p. 844-854.
267. Cohen, H.B., et al., *IFN-γ prevents adenosine receptor (A2bR) upregulation to sustain the macrophage activation response.* J Immunol, 2015. **195**(8): p. 3828-3837.
268. Gifford, G.E. and M.-L. Lohmann-Matthes, *Gamma interferon priming of mouse and human macrophages for induction of tumor necrosis factor production by bacterial lipopolysaccharide.* J Natl Cancer Inst 1987. **78**(1): p. 121-124.
269. Vila-del Sol, V., C. Punzón, and M. Fresno, *IFN-γ-induced TNF-α expression is regulated by interferon regulatory factors 1 and 8 in mouse macrophages.* J Immunol, 2008. **181**(7): p. 4461-4470.
270. Jun, C.D., et al., *Nitric oxide induces ADP-ribosylation of actin in murine macrophages: association with the inhibition of pseudopodia formation, phagocytic activity, and adherence on a laminin substratum.* Cell Immunol, 1996. **174**(1): p. 25-34.
271. Peppelenbosch, M., et al., *Multiple signal transduction pathways regulate TNF-induced actin reorganization in macrophages: inhibition of Cdc42-mediated filopodium formation by TNF.* J Immunol, 1999. **162**(2): p. 837-845.
272. Gordon, S.R., et al., *PD-1 expression by tumour-associated macrophages inhibits phagocytosis and tumour immunity.* Nature, 2017. **545**(7655): p. 495-499.
273. Aggarwal, B.B., *Signalling pathways of the TNF superfamily: a double-edged sword.* Nature Reviews Immunology, 2003. **3**(9): p. 745-756.
274. Cullen, G., et al., *Psoriasis associated with anti-tumour necrosis factor therapy in inflammatory bowel disease: a new series and a review of 120 cases from the literature.* Alimentary Pharmacology & Therapeutics, 2011. **34**(11-12): p. 1318-1327.

275. Zganiacz, A., et al., *TNF-α is a critical negative regulator of type 1 immune activation during intracellular bacterial infection.* Journal of Clinical Investigation, 2004. **113**(3): p. 401-13.
276. Moore, T.A., et al., *Defective innate antibacterial host responses during murine Klebsiella pneumoniae bacteremia: tumor necrosis factor (TNF) receptor 1 deficiency versus therapy with anti-TNF-alpha.* Clin Infect Dis, 2005. **41 Suppl 3**: p. S213-7.
277. Galván-Peña, S. and L.A. O'Neill, *Metabolic reprograming in macrophage polarization.* M1/M2 Macrophages: The Arginine Fork in the Road to Health and Disease, 2015. **5**(420): p. 275.
278. McPhillips, K., et al., *TNF-alpha inhibits macrophage clearance of apoptotic cells via cytosolic phospholipase A2 and oxidant-dependent mechanisms.* J Immunol, 2007. **178**(12): p. 8117-26.

## Appendix 1: Published articles

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Short communication

# The absence of TNF permits myeloid Arginase 1 expression in experimental MARK L. monocytogenes infection

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ABSTRACT

During an immune response inflammatory macrophages with their wide variety of effector mechanisms including the expression of inducible nitric oxide synthase play an important part in the defense against invading pathogens. The inflammatory phenotype requires the presence of TNF which suppresses alternative activation. In the bacterial Listeria monocytogenes infection model inflammatory macrophages are crucial for protection. After infection, TNF-deficient hosts have a similar number of splenic macrophages but die rapidly. A more detailed analysis of these cells showed that while inducible nitric oxide synthase is expressed at a comparable level TNF-deficient macrophages show an increased expression of Arginase 1.

## 1. Introduction

Failure to resolve inflammation is intrinsic in many human pathologies. Mechanisms that influence and modulate the inflammatory response are therefore, of general interest for our understanding of the underlying disease processes. One of the classical protagonists of inflammation is the proinflammatory cytokine tumor necrosis factor (TNF). This cytokine is present in high concentrations in inflammatory sites and has a variety of functions, such as activating cells and inducing the expression of functional cell surface molecules (Sedgwick et al., 2000). TNF is expressed by macrophages and T cells early after an immunological challenge and has been identified as a target for therapeutic intervention in a variety of chronic and autoimmune inflammatory diseases, including rheumatoid arthritis (RA) and inflammatory bowel disease (IBD) (Efimov et al., 2009; Udalova et al., 2016a). Anti TNF-therapy that blocks TNF activity using antagonists based on antibodies or TNFR fusion proteins is now a proven method of treatment for these pathologies (Udalova et al., 2016b). However, TNF has also been identified to be essential in the establishment of protective immunity to infection. Experiments that block TNF and the use of gene-deficient animals have resulted in a list of bacterial and parasitic pathogens that are controlled by a functioning TNFR1-TNF signaling axis (Pfeffer et al., 1993; Rothe et al., 1993; Wilhelm et al., 2001). The implicit mechanisms that contribute to the protective role of TNF have been difficult to define due to the ubiquitous expression of this pleiotropic cytokine. In the murine model of experimental cutaneous leishmaniasis, TNF-deficiency induced a progressive visceral infection and was ultimately fatal in the normally resistant mouse strain C57BL/6 (Wilhelm et al., 2001). The underlying cause of this lack of protection proved to be elusive since both innate and adaptive immune responses seem largely unchanged. However, it has recently been shown that TNF is essential for the suppression of Arginase 1 (Arg1) and other genes of the pro-homeostatic alternatively activated macrophage signature due to a restriction of accessibility of their promoters and enhancers (Schleicher et al., 2016). Thus, TNF was deemed irreplaceable for an effective differentiation of monocytes to classically activated macrophages (Schleicher et al., 2016). Indeed, a large percentage of skin and lymph node-resident macrophages and inflammatory dendritic cells (DC) from Leishmania (L.) major infected B6.TNF$^{-/-}$ mice co-expressed classically and alternatively activated macrophage signature molecules such as Arg1 and iNOS, respectively (Schleicher et al., 2016). This co-expression resulted in a lack of the central effector molecule nitric oxide (NO) in the lesion and the draining lymph node in infected tissues presumably due to a depletion of L-arginine, the substrate of both enzymes. This biological function of TNF to

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restrict alternative macrophage differentiation during the inflammatory response to L. major has also been demonstrated in tumor models and we hypothesized that it would be applicable generally.

Therefore, we used Listeria (L.) monocytogenes infection as a bacterial model with a well-established history of myeloid differentiation to analyze the consequences of TNF-deficiency in more detail (Serbina et al., 2003). The TNF-deficient mouse strain (B6.TNF$^{-/-}$) was highly susceptible to the pathogen. Inflammatory splenic macrophages from B6. TNF$^{-/-}$ mice could be identified using Ly6 C and CD11b. These cells exhibited a high bacterial burden, normal iNOS and an elevated Arg1 and TGM2 expression.

## 2. Material and methods

### 2.1. Animals and infection

Eight to 16-week-old C57BL/6 (B6. WT) and B6.TNF$^{-/-}$ mice were used in all experiments. All mice were housed and bred in a certified SPF environment. To infect the mice we cultured a single colony of L. monocytogenes (obtained from Prof. Dirk Busch, Institute for Microbiology, Technical University Munich, Germany) in brain-heart infusion broth (BD Biosciences, North Ryde, Australia) overnight at 37 °C with shaking. The overnight culture was added to fresh medium at volume ratio 1: 50 and cultured for 1 h with shaking until an OD600 of 0.1 was reached. In a volume of 100 ul PBS 1000 L. monocytogenes were injected intraperitoneally. All animal procedures were approved by the Animal Ethics Committee of the University of Tasmania (permit number A13933).

### 2.2. Flow cytometry analysis

Spleens were dissociated with collagenase V (Sigma-Aldrich, Castle Hill, Australia) and red blood cells lysed using a Hepes-buffered ammonium chloride lysis buffer. Splenocytes were filtered through cell strainers (40 μm; BD Biosciences) to remove cell debris. Cells were stained with rat anti-mouse mAbs against Ly6 C (AL-21, FITC, BD Biosciences), CD11b (M1/70, PerCP-Cy5.5, BD Biosciences), CD11c (HL3, PE-Cy7, BD Biosciences), Ly-6G (IA8, PE, BD Biosciences), Ly-6G (IA8, BV421, Biolegend, San Diego, USA), The rat anti-mouse CCR2 mAb (MC21) has been described (Mack et al., 2001) and was provided by Prof. Matthias Mack (Universitaetsklinikum Regensburg, Regensburg, Germany). The CCR2 antibody was used unlabeled and before addition of donkey anti-rat AF647 (Jackson ImmunoResearch Laboratories, West Grove, USA) as secondary antibody cells were blocked with 10% rat serum (Sigma-Aldrich) for 10 min on ice.

Dead cells were excluded by DAPI (Sigma-Aldrich). Data were acquired using CyAn ADP (Beckman Coulter, Gladesville, Australia) and data analysis was performed using FlowJo V10.1 (Tree Star, USA). Gating controls were determined by fluorescence minus one (FMO) controls. For flow cytometric cell sorting we used a MoFlo Astrios cell sorter (Beckman Coulter). The purity obtained was > 95%.

### 2.3. CFUs assay

Spleen and liver were removed from animals and homogenized in PBS containing 0.05% Triton X-100 (Sigma-Aldrich). Alternatively, Ly6 C$^{+}$ macrophages were isolated by flow cytometry and 5000 cells were lysed in the same buffer. Serial dilutions were plated onto brainheart infusion agar plates and colony forming units (CFUs) counted after overnight culture at 37 °C. CFU were recorded as CFU/organ or CFU/5000 cells.

### 2.4. Quantitative real-time PCR

Splenic Ly6 C$^{+}$ monocytes were sorted as described. RNA was extracted using ReliaPrep™ RNA Cell Miniprep System (Promega, Sydney, Australia) according to the manufacturer's instructions. The iScript™ gDNA Clear cDNA Synthesis Kit (Bio-Rad, Gladesville, Australia) was used to reverse-transcribe total RNA. Quantitative real-

Table 1 qPCR primers used in this study.

| Primers | Forward 5′-3′ | Reverse 5′-3′ | Product length |
|---|---|---|---|
| Arg1 | GTG AAG AAC CCA CGG TCT GT | CTG GTT GTC AGG GGA GTG TT | 209bp |
| GAPDH | GTG AAG GTC GGT GTG AAC GG | ATG TTA GTG GGG TCT CGC TCC | 245bp |
| iNOS | GGA ATC TTG GAG CGA GTT GT | CCT CTT GTC TTT GAC CCA GTA G | 99bp |
| TGM2 | CGA ATC CTC TAC GAG AAG TAC AGC | CAG TTT GCG GTT TTG CTT GG | 177bp |

time PCR (qPCR) was carried out on a Roche Lightcycler 480 qPCR instrument (Roche, North Ryde, Australia) with 10 μl reactions using the SsoAdvanced™ Universal SYBR® Green Mix (Bio-Rad). Primers are listed in Table 1. Reactions were performed in duplicate and gene expression levels were normalized to the housekeeping gene GAPDH.

### 2.5. Statistical analysis

Statistical analyses were performed using GraphPad Prism 5 software. Statistical significance was determined by Mann–Whitney U test. Data were shown as the mean ± SEM. P values of smaller than 0.05 were considered statistically significant.

## 3. Results

The experimental infection of mice with L. monocytogenes is a wellvalidated model for a bacterial infection that relies on macrophages as predominant host cells. Spleen and liver are the two major target organs of systemic L. monocytogenes infection. In B6.WT mice the infection reached a peak of $10^4$ bacteria per organ at day 3 post-infection (p.i.) and started to subside thereafter (Fig. 1A and B). At the same time post infection, in spleens and livers of immunocompromised B6.TNF$^{-/-}$ mice the number of L. monocytogenes exceeded $10^6$ and $10^5$, respectively, and continued to increase at a dramatic rate (Day4: > $10^7$ in spleen and liver).

Based on a Ly6G$^-$ CD11b$^+$ CD11c$^+$ phenotype, two major populations of myeloid cells (Ly6G$^-$ CD11b$^{high}$ CD11c$^{low}$ (R1) and Ly6G$^-$ CD11b$^{low}$ CD11c$^{high}$ (R2)) could be readily identified in the spleens of B6.WT and B6.TNF$^{-/-}$ mice three days after infection. Further analysis showed that the Ly6G$^-$ CD11b$^{high}$ CD11c$^{low}$ cells displayed an inflammatory phenotype with a strong expression of CCR2 and Ly6 C, Fig. 1C) while cells of the population R2 were predominately negative for CCR2 and Ly6 C (Fig. 1C). Subsequently, the development of the population of Ly6G$^-$ CD11b$^{high}$ CD11c$^{low}$ CCR2$^+$ Ly6 C$^+$ macrophages was analyzed in a time course. After infection, the proportion of inflammatory cells increased rapidly reaching an observed peak at day 4 after infection (Fig. 1D). Because of the susceptibility of the B6.TNF$^{-/-}$ mice to the pathogen the experiment had to be stopped at this stage. Interestingly, the total number while also significantly increasing from 1 × $10^6$ (day 0 p.i.) to 2.6 × $10^6$ (day 2 p.i.) did not differ significantly between the genotypes (Fig. 1E).

An analysis of inflammatory macrophages sorted from B6.WT and B6.TNF$^{-/-}$ mice at day 3 post infection showed a significant presence of bacteria in B6.TNF$^{-/-}$ macrophages while B6.WT were virtually free of pathogens indicating a lack of intrinsic defense mechanisms in the TNF knockout strain (Fig. 2A). Therefore, we investigated the expression of the classically activated macrophage marker iNOS at this point in the infection and in parallel, Arg1 and TGM2 as marker molecules for alternative activation. Despite the absence of TNF, iNOS expression was unchanged in both genotypes pointing to a lack of TNF-dependent regulation of this enzyme (Fig. 2B). In contrast, the alternative activation marker

molecules Arg1 and TGM2 were significantly upregulated in B6.TNF$^{-/-}$ macrophages (Fig. 2C, D). These observations

are in agreement with earlier findings in the model of experimental leishmaniasis (Schleicher et al., 2016; Fromm et al., 2012). Other marker molecules such as Fizz1 were not affected by TNF-deficiency and were equally expressed in both genotypes (data not shown).

## 4. Discussion

A deficiency in the TNF-TNFR1 signaling pathway causes a significant aggravation of the pathology of C57BL/6 mice infected with L. monocytogenes (Grivennikov et al., 2005; Pfeffer et al., 1993; Rothe et al., 1993). Investigations using a tissue-specific TNF-deficiency have been able to localize the source of the protective production of TNF in macrophages (Grivennikov et al., 2005). The TNF-deficient strain B6.TNF$^{-/-}$ displayed a rapid increase of the bacterial burden in spleen and liver within 4 d after intraperitoneal infection with 1000 CFU while B6. WT mice were able to control the infection. Interestingly, an analysis of the expression of the enzymes iNOS, Arg1 and TGM2, marker molecules for classically and alternatively activated macrophages respectively, by splenic macrophages showed a significant presence of the enzymes Arg1 and TGM2 which are considered to be marker for alternative activation.

This is an important finding since a recent detailed analysis of the innate immune response to the parasite L. major has demonstrated a skewed development of macrophages to an alternative activation-like phenotype in the absence of TNF (Schleicher et al., 2016). This newly described pro-inflammatory function of the TNF-TNFR1 axis has been supported by observations in two tumor models (Kratochvill et al., 2015). In the absence of TNFR1 an emergence of alternatively activated macrophages was reported. For the first time, our investigation of the listeriosis model now extends these findings to bacterial infection. This confirms the biological relevance of the initial reports. Our findings are highly significant because the ubiquitous presence and the high density of TLR-ligands in bacterial infection could have been instrumental in overriding the necessity for the pro-inflammatory role of TNF which suppresses Arg1 expression. Our data clearly indicate that TNF negatively regulates Arg1 expression irrespective of the inflammatory environment in the host.

A second important aspect of our observation is the increasing evidence for a general mechanism underlying the general susceptibility to intracellular pathogens of mice deficient for the TNF-TNFR1-signaling pathway. In the analysis of the L. major infection model a competitive co-expression of iNOS and Arg1 could be demonstrated that caused a significant decrease of NO. This effector molecule is central in macrophage defense against intracellular infection (Bogdan et al., 2000) and a co-expression of both enzymes could deplete the pool of the substrate L-arginine and cause a lack of NO (Schleicher et al., 2016). This mechanistic aspect needs further investigation.

Taken together, we have demonstrated a role for TNF in the development of classically activated macrophages in listeriosis. Without TNF, we demonstrated an increased production of Arg1, a hallmark of alternative activation in mice (Murray et al., 2014). Further research will be necessary to identify the pathways impacted by the lack of TNF and for proof of the involvement of L-arginine depletion for the augmented susceptibility to L. monocytogenes.

## Conflict of interest

The authors state that they had no conflict of interest.

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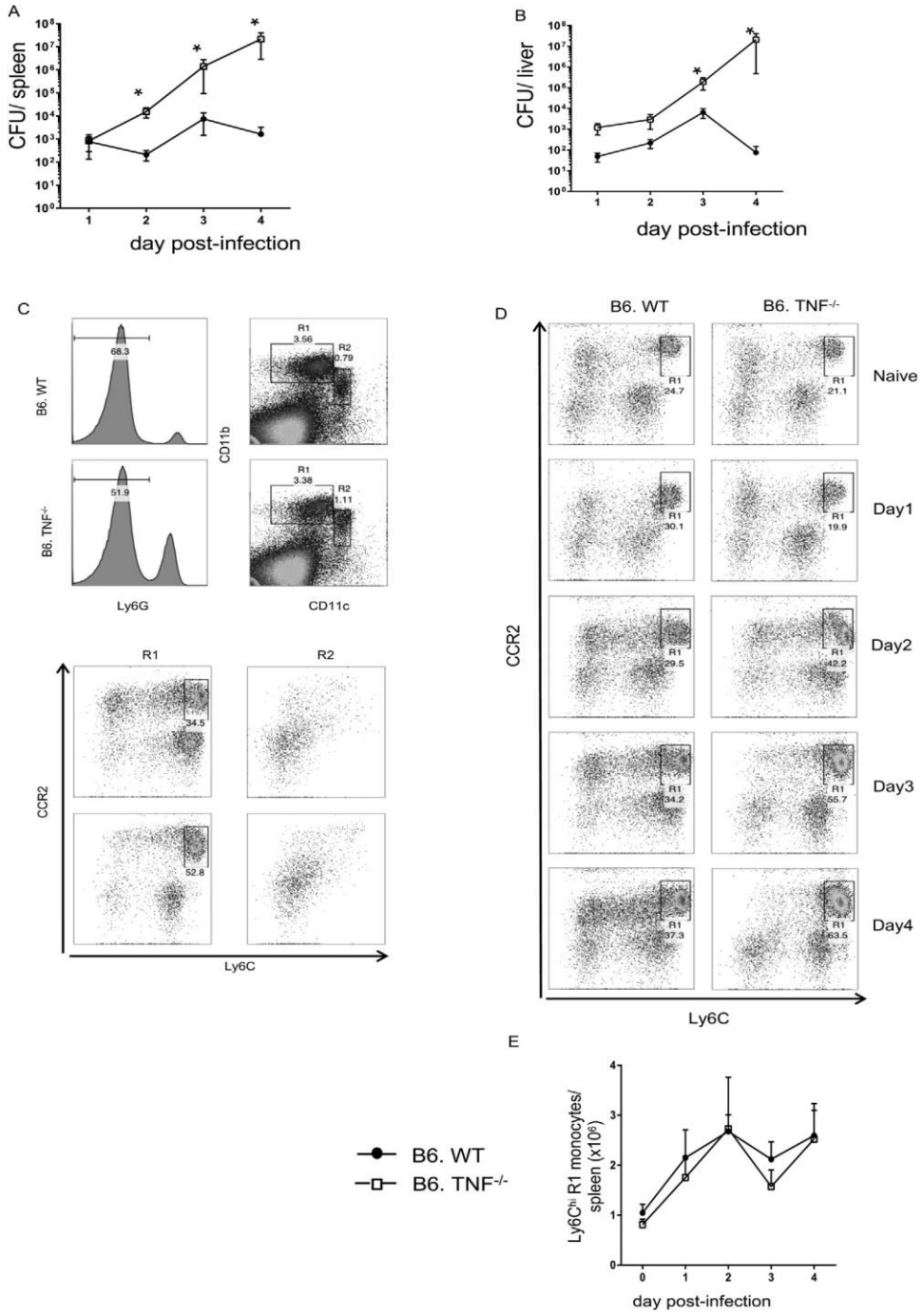


Fig. 1. TNF deficiency causes susceptibility to L. monocytogenes infection but does not change splenic Ly6 $C^{hi}$ monocyte recruitment. B6.WT and B6.$TNF^{-/-}$ mice were infected with 1000 L. monocytogenes intraperitoneally. Bacterial load and number of splenic monocytes were analyzed. (A,B) Bacterial load in spleen and liver of B6. WT and B6.$TNF^{-/-}$ mice was determined by culturing organ homogenates on BHI plates overnight (n = 4–6 mice in two independent experiments). The results are shown as mean ± SEM, *p < 0.05. (C) Representative FACS plots using Ly6G (granulozytes), CD11b and c, and CCR2 and Ly6 C. (D) Representative FACS plots of splenic $CD11b^{+}CCR2^{+}$Ly6 $C^{+}$ monocytes from control

(uninfected) or L. monocytogenes-infected B6.WT and B6.TNF$^{-/-}$ mice over a period of 4 d after infection. (E) Quantification of total numbers of CD11b$^{+}$CCR2$^{+}$ Ly6 C$^{+}$ monocyte from uninfected or L. monocytogenes infected B6.WT and B6.TNF$^{-/-}$ mice (n = 6–12 mice from two or three independent experiments). The results are shown as mean ± SEM.

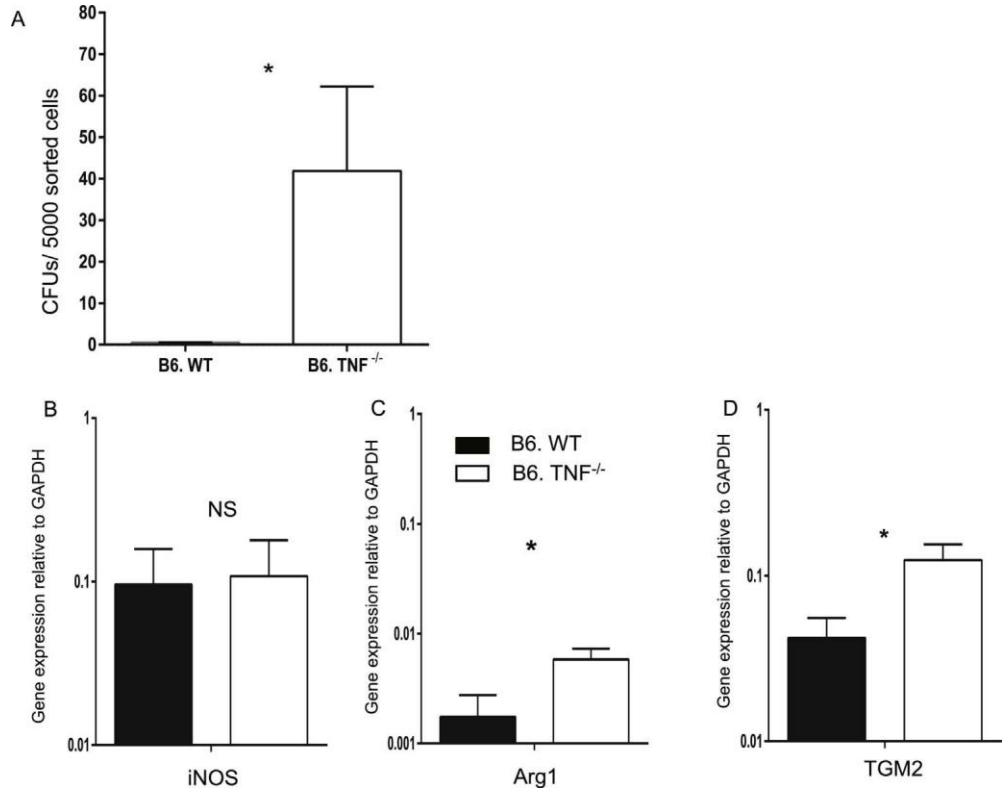


Fig. 2. Lack of TNF impairs anti- L. monocytogenes function and leads to the M2 phenotype of Ly6 C$^{+}$ monocytes during L. monocytogenes infection. B6.WT and B6.TNF$^{-/-}$ mice were infected with 1000 L. monocytogenes intraperitoneally. Ly6 C$^{+}$ monocytes were sorted from B6.WT and B6.TNF$^{-/-}$ mice infected with L. monocytogenes for 3 d. (A) Bacterial loads in 5000 Ly6 C$^{+}$ monocytes were measured using BHI agar plates (n = 6 from two independent experiments). (B–D) The expression of iNOS, Arg1 and TGM2 mRNA in Ly6 C$^{+}$ R1 monocytes from B6.WT and B6.TNF$^{-/-}$ mice were assessed by qPCR (primer see Table 1) (n = 7–8 mice from two independent experiments). The results are shown as mean ± SEM, *p < 0.05.

References

Bogdan, C., Röllinghoff, M., Diefenbach, A., 2000. The role of nitric oxide in innate immunity. Immunol. Rev. 173, 17–26.

Efimov, G.A., Kruglov, A.A., Tillib, S.V., et al., 2009. Tumor necrosisfactor and the consequences of its ablation in vivo. Mol.Immunol. 47, 19–27.

Fromm, P.D., Kling, J., Mack, M., et al., 2012. Loss of TNF signaling facilitates the development of a novel Ly-6C(low) macrophage population permissive for Leishmania major infection. J.Immunol. 188, 6258–6266.

Grivennikov, S.I., Tumanov, A.V., Liepinsh, D.J., et al., 2005. Distinct and nonredundant in vivo functions of TNF produced by T cells and macrophages/neutrophils: protective and deleterious effects. Immunity 22, 93–104.

Kratochvill, F., Neale, G., Haverkamp, J.M., et al., 2015. TNF counterbalances the emergence of M2 tumor macrophages. Cell.Rep. 12, 1902–1914.

Mack, M., Cihak, J., Simonis, C., et al., 2001. Expression and characterization of the chemokine receptors CCR2 and CCR5 in mice. J. Immunol. 166, 4697–4704.

Murray, P.J., Allen, J.E., Biswas, S.K., et al., 2014. Macrophage activation and polarization: nomenclature and experimental guidelines. Immunity 41, 14–20.

Pfeffer, K., Matsuyama, T., K¸ndig, T.M., et al., 1993. Mice deficient for the 55 kd tumor necrosis factor receptor are resistant to endotoxic shock, yet succumb to L. monocytogenes infection. Cell 73, 457–467.

Rothe, J., Lesslauer, W., Lotscher, H., et al., 1993. Mice lacking the tumour necrosis factor receptor 1 are resistant to IMF-mediated toxicity but highly susceptible to infection by Listeria monocytogenes. Nature 364, 798–802.

Schleicher, U., Paduch, K., Debus, A., et al., 2016. TNF-mediated restriction of Arginase 1 expression in myeloid cells triggers type 2 NO synthase activity at the site of infection. Cell. Rep. 15, 1062–1075.

Sedgwick, J.D., Riminton, D.S., Cyster, J.G., et al., 2000. Tumor necrosis factor: a masterregulator of leukocyte movement. Immunol. Today 21, 110–113.

Serbina, N.V., Salazar-Mather, T.P., Biron, C.A., et al., 2003. TNF/iNOS-producing dendritic cells mediate innate immune defense against bacterial infection. Immunity 19, 59–70.

Udalova, I.A., Mantovani, A., Feldmann, M., 2016a. Macrophage heterogeneity in the context of rheumatoid arthritis. Nat. Rev. Rheumatol. 12, 472–485.

Udalova, I., Monaco, C., Nanchahal, J., et al., 2016b. Anti-TNF therapy. Microbiol. Spectr. 4.

Wilhelm, P., Ritter, U., Labbow, S., et al., 2001. Rapidly fatal leishmaniasis in resistant C57BL/6 mice lacking TNF. J. Immunol. 166, 4012–4019.

## Appendix 2: Manuscript for publication

**TNF signalling downregulates phagocytosis of tumour cells by activated macrophages**

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## Abstract

Macrophage phagocytosis of pathogens and tumour cells is an important early event in protection against infectious disease and cancer. As tumour necrosis factor α (TNF) is an important cytokine in macrophage activation, we investigated the involvement of TNF in macrophage phagocytosis of tumour cells. We used Devil Facial Tumour Disease (DFTD) cancer cells as the target tumour cells. The Tasmanian devil *(Sarcophilus harrisii*) population is threatened by the transmissible DFTD. By using DFTD cells provided the opportunity to determine if these cells can be phagocytosed and investigate requirement for TNF. If DFTD cells are unable to be phagocytosed, this might be another immune escape mechanism. As effector cells, bone marrow derived macrophages (BMDMs), generated from C57BL/6 wild type (B6.WT) and C57BL/6 TNF$^{-/-}$ (B6.TNF$^{-/-}$) mice were used. Phagocytosis of DFTD cells was investigated by confocal microscopy and flow cytometry. DFTD cells were effectively phagocytosed by B6.WT and B6.TNF$^{-/-}$ BMDMs with similar efficiency. Consequently the DFTD cells are not resistant to phagocytosis. Following activation by exposure to IFNγ and LPS or LPS alone, B6.TNF$^{-/-}$ BMDMs had higher phagocytic efficiency and lower nitric oxide production compared to wild-type controls. In addition, nitric oxide (NO) inhibition failed to alter phagocytosis efficiency in IFNγ and LPS activated B6.TNF$^{-/-}$ macrophages. Our results indicate that TNF is not required for IFNγ and LPS or LPS alone activation of macrophage phagocytosis. TNF may negatively regulate macrophage phagocytosis of tumour cells.

## Introduction

Tumour necrosis factor α (TNF) is a member of the tumour necrosis factor family including TNF, TNFβ and lymphotoxin (LT) [245]. TNF receptor 1 (TNFR1) and TNFR2 are receptors for TNF [1]. Under steady-state conditions TNF is synthesised at low levels, but following infection macrophages increase the production of TNF [1]. Macrophages are a heterogeneous population of innate immune cells with the important role of protection against pathogens. Macrophage phagocytosis involves receptor recognition, cytoskeleton rearrangement and phagosome maturation [2]. During the process of phagocytosis pathogens are endocytosed and become engulfed as membrane coated phagosomes [3]. The phagosomes then fuse with lysosome to form an acidic environment to degrade the pathogens [2]. Actin polymerization is required for pathogen uptake and reactive oxygen and nitrogen species contribute to the degradation of the pathogen [2].

In response to different stimuli, macrophages differentiate into "classically activated macrophages (M1)" or "alternatively activated macrophages (M2)" [4]. M1 macrophages require exposure to the pro-inflammatory cytokines interferon gamma (IFNγ) either alone or in combination with TNF or LPS. M2 macrophages require exposure to IL-4 and/or IL-13 [4]. M1 macrophages promote inflammation whereas M2 macrophages are anti-inflammatory [5]. Furthermore, M1 and M2 macrophages display different levels of phagocytosis [5]. IFNγ activation of M1 macrophages enhances phagocytosis of apoptotic cells [6]. In contrast, IL-4 induced M2 macrophages exhibit impaired bacterial phagocytosis due to reduced phagosome formation [7]. TNF appears to inhibit macrophage phagocytosis of apoptotic cells in a time and dose dependent manner [8]. TNF deficient macrophages have a reduced ability to endocytose nontypeable *Haemophilus influenza* [9]. However, TNF appears to enhance IFNγ primed macrophage phagocytosis of apoptotic cells in a NO dependent manner [6]. Therefore, the effects of TNF in macrophage phagocytosis under different activation states of macrophages are unclear. Consequently, we investigated role of TNF in macrophage phagocytosis of tumour cells under different activation conditions. The tumour cells used in this study were Devil Facial Tumour Disease (DFTD) cells. These tumour cells were selected to determine if DFTD cells could be phagocytosed.

The first case of Devil Facial Tumour Disease was recorded in 1996 and is primarily responsible for the catastrophic decline of the Tasmanian devil (*Sarcophilus harrisii*) population. In 2014 a second and independent DFTD was discovered (Pye) and has been called DFT2 to distinguish it from the first DFTD, now identified as DFT1. DFTD is a

transmissible cancer transmitted as an allograft by biting and is characterized by tumours located on face and neck of diseased devils [10]. Functional and histological analyses performed to date indicate that the devil has a competent adaptive immune system [11]. Thus, generalised immunosuppression does not explain why the DFTD cancer cells are not immunologically rejected. The most likely explanation why DFTD escapes immune recognition is because the DFTD cancer cells do not express surface MHC-I [12]. This avoids activation of the specific immune system. It is unknown whether DFTD cells can be phagocytosed, hence the selection of these cells for this study.

We firstly determined if DFTD cells could be phagocytosed then examined different activation status of macrophages and the effect of TNF on their ability to phagocytose DFTD cells. We used bone marrow derived macrophages (BMDMs) isolated from wild type and B6.TNF$^{-/-}$ mice and found that TNF inhibits macrophage phagocytosis efficiency following activation by IFNγ/LPS and LPS.

## Materials and Methods

### Animals

The gene-targeted C57BL/6 (B6.TNF$^{-/-}$) mouse strains deficient for TNF were generated on a genetically pure C57BL/6 (B6.WT) background as described [13]. The screening procedure followed the protocols described previously [13]. All animals were housed in pathogen-free conditions. Mice aged 8-16 weeks were used in all experiments following approval of the Animal Ethics Committee of University of Tasmania (UTAS) under the ethics number A13934.

### Cell culture

Bone marrow derived macrophages (BMDMs) from B6.WT and B6.TNF$^{-/-}$ mice were generated from pelvic and femoral bones. BMDMs were cultured in RPMI 1640 medium (Gibco, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco, USA), 10% L929-conditioned medium (containing M-CSF), 5% horse serum (Gibco, USA), 1% penicillin and streptomycin (Gibco, USA), 1% nonessential amino acids (Gibco, USA) and 2-mercaptotoethanol (Gibco, USA). After culture for 7 days, BMDMs were untreated or treated with the following substances: 20 ng/ml IFNγ (Peprotech, USA), 100 ng/ml LPS

(Sigma, USA) or a combination of IFNγ and LPS for 24 hours or 5 ng/ml IL-4 (Peprotech, USA) for 48 hours. The purity of macrophages obtained was always at least 90%.

The Devil Facial Tumour Disease cell line, C5065, was established from primary tumour samples [14]. Cells were grown in RPMI-1640 medium (Gibco, USA) supplemented with 10% FBS (Bovogen Biological, Australia) and 5mM L-glutamine (Sigma, USA). DFTD cells were maintained in a humidified 5% $CO_2$ incubator at 35 °C. Phagocytosis assays required coculturing of mouse macrophages with DFTD cells were maintained at 37 °C.

**Flow cytometry phagocytosis assay and calculations**

BMDMs (effector cells) were labelled with CellTrace™ Violet (CTV, Invitrogen, USA), DFTD cells (target cells) were labelled with 5(6)-Carboxyfluorescein diacetate Nsuccinimidyl ester (CFSE, Invitrogen, USA) according to manufacturer's instruction. BMDMs were resuspended in cell culture medium at $10^6$/ml. Serial dilution of 100 µl BMDMs were performed to obtain effector to target ratios of 10:1, 5:1 with $10^4$ target cells. The phagocytosis assays were performed using duplicates or triplicates and incubated for 4 hours, 24 hours at 37 °C and for 4 hours at 4 °C for negative control. The plates were analysed on a BD Canto II flow cytometer and data were analysed with Flowjo V10.1 software (Tree star, USA), and Graphpad Prism 5 for graphic display. To calculate phagocytosis, three gates were identified. R1, total $CFSE^+$ tumour cells = total tumour cells. R2, $CFSE^+/CTV^+$ cells = tumour cells phagocytized by macrophages. The formula used for calculating phagocytosis was: percentage of phagocytosis= R2/R1 x100. For the 24 hours incubation (to avoid complication of cell division), the absolute numbers of DFTD cells were calculated by including all events (i.e. cells) or enumerating with a known number of cell count beads (eBioscience, USA). For the bead count method, the number of DFTD cells = (number of beads added/ number of beads collected) x number of $CFSE^+$ $CTV^-$ DFTD cells.

**Cell staining and confocal microscopy for evidence of phagocytosis**

CTV labelled BMDMs were grown in 24 well plates on 0.2% gelatine (BD Bioscience, USA) coated 12 mm coverslips. CFSE labelled DFTD cells were added when the coverslip was almost confluent with BMDMs. After 4 and 24 hours incubation, cells were gently washed with PBS. Cells were permeabilized with 0.25% Triton X-100 (Sigma, USA) for 20 min and blocked with a 1% BSA (Sigma, USA) in PBS solution for 20 min. Antibody staining was conducted for 30 min with a rabbit anti-mouse-LAMP1 antibody (BD Bioscience, USA). The goat anti rabbit Alexa Fluor 594 (BD Pharmingen, USA) secondary antibody was applied for

30 min. The cells were washed three times in PBS and mounted using Dako mounting medium (DakoCytomation, Australia). Confocal microscopy was performed using spinning disk and z-stacks were taken in slices of 1 µm.

**Griess assay**

The contents of nitrate and nitrite in the cell culture media were determined by Griess assay as described previously [15]. 50 µl cell culture medium was mixed with equal volumes of Griess reagents (1% sulphanilamide in 2.5% $H_3PO_4$, Sigma, USA), then 50 µl Griess reagent (0.1% napthylenediamide dihydrochloride, Sigma, USA) was added and incubated at room temperature for 10 min. The absorbance at 540 nm was measured by a microplate reader (Bio-Rad, USA) and compared to a standard nitrite curve using sodium nitrite (Sigma, USA).

**Statistical analysis**

Flow cytometry was conducted at least three times in duplicate or triplicate wells. Statistical significance was determined by Student's unpaired t-test. P values of less than 0.05 were considered statistically significant.

**Results**

**DFTD cells can be phagocytosed by B6.WT and B6.TNF$^{-/-}$ macrophages.**

To determine if DFTD cells could be phagocytosed, BMDMs from B6.WT and B6.TNF$^{-/-}$ mice (CTV labelled; blue) were co-incubated with DFTD C5065 cells (CFSE-labelled; green). After 4 hours and 24 hours culture, phagocytosis was analysed by confocal microscopy (Figure 1). Green DFTD cells could be identified within the blue macrophages from both B6.WT and B6.TNF$^{-/-}$ mice. When *z*-stacks were analysed the green fragments were identified inside the macrophages, suggesting the tumour cells had been engulfed (Fig 1). The degradation of phagocytosed cellular material involves the process of phagosome maturation through homotypic and heterotypic fusion of early endosomes, late endosomes and lysosomes [16]. The presence of lysosomal-associated membrane protein 1 (LAMP1) was used to discriminate a late phase of phagocytosis, after phagosomes/lysosome fusion [17]. LAMP1 and engulfed tumour fragments were co-localized with LAMP1, confirming phagocytosis. As BMDMs from B6.WT and B6.TNF$^{-/-}$ mice can phagocytose DFTD cells, it suggests that TNF is not essential for phagocytosis and that DFTD cells are not resistant to phagocytosis.

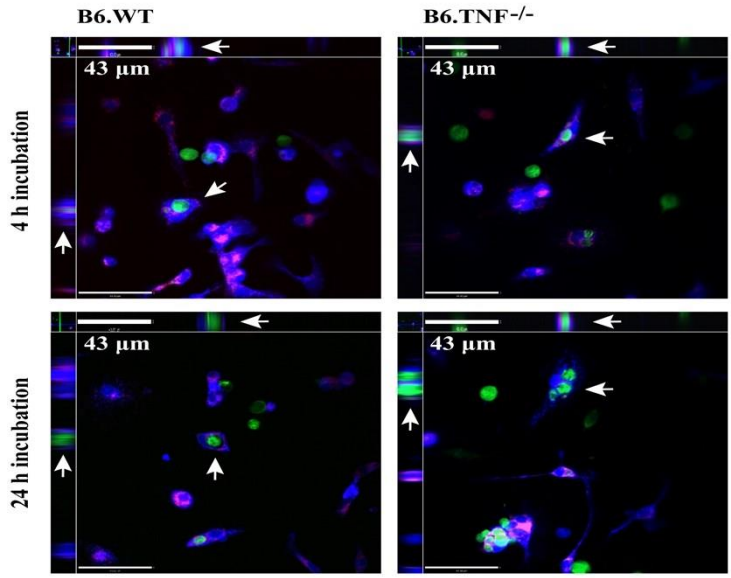


**Figure 1. Confocal microscope analysis of macrophage phagocytosing DFTD cells.** Co-incubate CTV labelled BMDMs with CFSE labelled DFTD cells for 4 hours and 24 hours, cells were fixed and permeabilized, then labelled with anti-LAMP1 antibody. Phagocytosis was analysed in confocal microscopy. The z-stack demonstrated that tumour cells inside B6.WT and B6.TNF$^{-/-}$ macrophages after 4 hours and 24 hours incubation (Green, DFTD cells; Blue, macrophages; Red, LAMP1).

**TNF deficiency results in reduced phagocytic capacity in IFNγ/LPS treated macrophages.**

Confocal microscopy showed that DFTD cells are phagocytosed by BMDMs from B6.WT and B6.TNF$^{-/-}$ mice, but to determine phagocytic efficiency flow cytometry was used. BMDMs were labelled with CTV and DFTD cells were labelled with CFSE, then incubated at ratios of BMDMs: DFTD cells (10:1 and 5:1) at 37 ºC or 4 ºC for 4 hours. The phagocytosed DFTD cells were recognized as CFSE and CTV double positive cells. In the absence of stimulation (control) the phagocytic efficiencies of BMDMs from B6.WT and B6.TNF$^{-/-}$ mice were similar at both 10:1 (Fig 2A) and 5:1 (Fig 2B) ratios, indicating that DFTD cells can be phagocytosed. It also suggests that TNF is not required for macrophage phagocytosis of DFTD cells under steady-state conditions.

In order to determine TNF involvement in phagocytosis following macrophage activation,

BMDMs were stimulated with IFNγ and LPS, IFNγ alone, LPS alone or IL-4 alone. Following IFNγ/LPS activation, macrophages from B6.TNF$^{-/-}$ mice displayed enhanced phagocytosis efficiency at both 10:1 (Fig 2A) and 5:1 (Fig 2B) ratios, compared with macrophages from B6.WT mice. However, BMDM macrophages from B6.TNF$^{-/-}$ mice stimulated with IFNγ or IL-4 did not show a significant difference in phagocytic efficiency, compared with BMDM from B6.WT mice (Fig 2A, 2B). Compared to untreated BMDMs, LPS treatment enhanced the phagocytic efficiency of BMDMs from wild-type and B6.TNF$^{-/-}$ mice (Fig 2A, 2B). But no significant difference was shown between BMDMs from wildtype and B6.TNF$^{-/-}$ mice following LPS activation.

To confirm that the CFSE and CTV double positive cells represented phagocytosis rather than cell binding, DFTD cells and macrophages were co-incubated at 4 ºC for 4 hours (Fig 2C, 2D). The proportion of double positive cells was substantially reduced providing support for phagocytosis at 37 ºC rather than just binding to the cell surface.

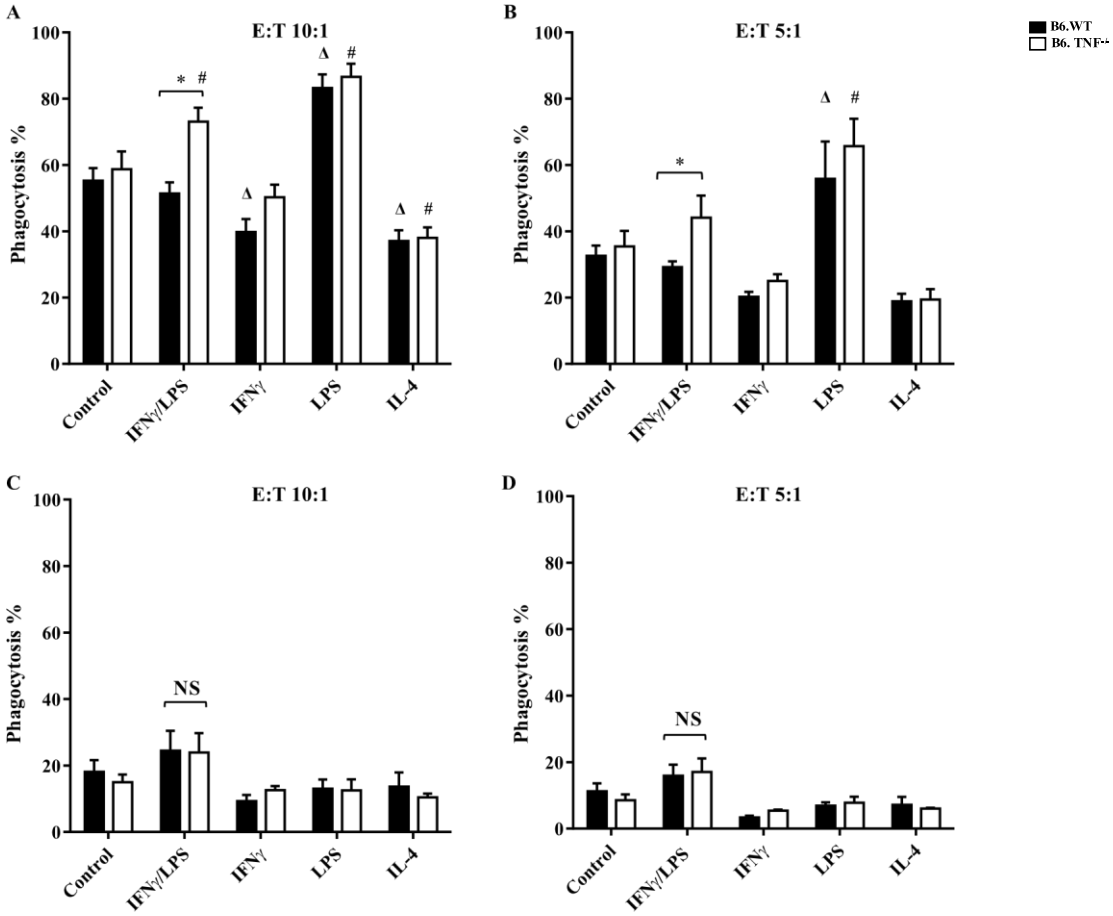


**Figure 2. IFNγ/LPS treated B6.TNF-/- BMDMs exhibited enhanced phagocytic efficiency of DFTD cells compared to B6.WT BMDMs.** B6.WT and B6.TNF$^{-/-}$ BMDMs were treated with IFNγ/LPS, IFNγ alone, LPS alone, or IL-4 alone. BMDMs

were labelled with CTV and DFTD cells were labelled with CFSE. After labelling, BMDMs were co-incubated with DFTD cells for 4 hours. The percentage of phagocytosed DFTD cells in the incubation of 37 °C (A, B) and 4 °C (C, D) was investigated by flow cytometry. Data were from 3-7 mice of two or three independent experiments, error bars indicate SEM, ΔP< 0.05 versus WT control, #P< 0.05 versus B6.TNF$^{-/-}$ control, *P< 0.05 versus WT versus B6.TNF$^{-/-}$, NS not significantly.

**IFNγ/LPS, LPS activated BMDM from B6.TNF$^{-/-}$ mice phagocytose and kill DFTD cells more effectively than BMDM from B6.WT mice following 24 hours incubation.**

DFTD cell phagocytosis and killing was analysed with flow cytometry after 24 hours incubation by enumerating the number of viable DFTD cells remaining. The absolute number of free DFTD cells was calculated using cell count beads. IFNγ/LPS and LPS alone activated BMDMs from B6.TNF$^{-/-}$ mice cultured with DFTD cells for 24 hours resulted in fewer DFTD cells remaining compared to BMDMs from B6.WT mice at 10:1 and 5:1 ratios (Fig 3A, 3B). However, IFNγ or IL-4 activated BMDMs from B6.TNF$^{-/-}$ mice had similar number of DFTD cells with wild-type controls at 10:1 and 5:1 ratios (Fig 3A, 3B). These results indicate that LPS and potentially IFNγ/LPS activated macrophages from B6.TNF$^{-/-}$ mice phagocytosed more DFTD cells than macrophages from the B6.WT mice. Thus, although phagocytosis by activated BMDMs was observed in the presence of TNF, the absence of TNF appeared to increase the phagocytic activity.

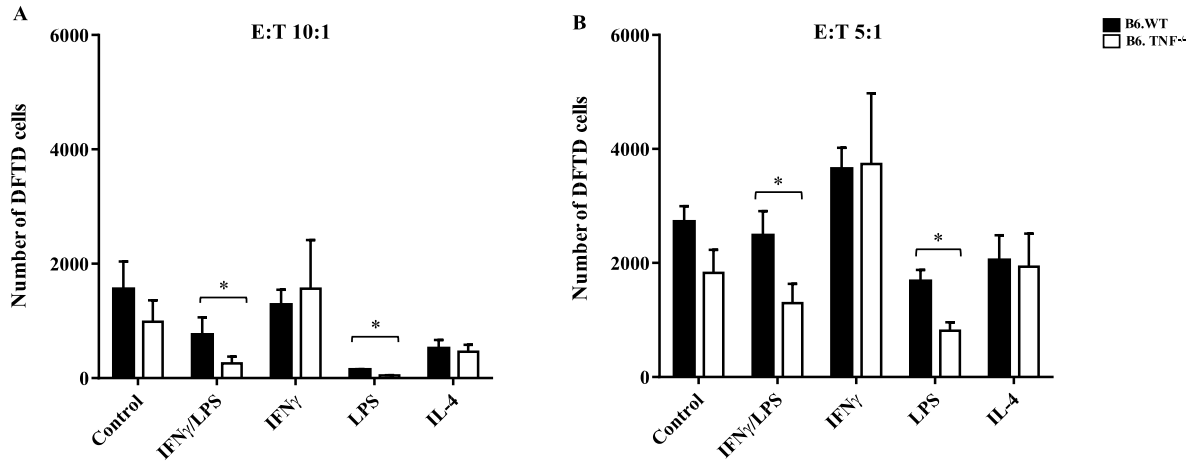

**Figure 3: IFNγ/LPS, LPS activated B6.TNF$^{-/-}$ BMDMs are more effective at removing DFTD cells after 24 hours incubation than B6.WT BMDMs.** BMDMs from B6.WT and B6.TNF$^{-/-}$ mice were treated with IFNγ/LPS, IFNγ alone, LPS alone, or IL-4 alone. BMDMs were labelled with CTV and DFTD cells were labelled with CFSE. After labelling, BMDMs were incubated with DFTD cells for 24 hours, and the absolute number of free DFTD cells was calculated using flow cytometry with cell count beads. Data were from 3-7 mice of two or three independent experiments, error bars indicate SEM, * P< 0.05.

**Less nitric oxide is released from IFNγ/LPS, LPS activated B6.TNF$^{-/-}$ macrophages after incubation with DFTD cells for 24 hours.**

Nitric oxide is an essential effector molecule in macrophages and is cytotoxic to tumour cells [18]. As TNF regulates the production of NO in macrophages [19], we determined whether NO is related to TNF regulation of macrophage phagocytosis of DFTD cells. IFNγ/LPS, IFNγ or LPS treated macrophages from B6.WT, B6.TNF$^{-/-}$ mice were co-incubated with DFTD cells for 24 hours and the NO contents in the cell culture supernatant were measured. As shown in Fig 4, untreated macrophages from both mouse genotypes did not release any detectable NO. In response to IFNγ/LPS activation, BMDMs from B6.TNF$^{-/-}$ mice released less NO than wild type controls. This was evident at 10:1 (Fig 4A) and 5:1 (Fig 4B) ratios. Similarly, less NO was released from LPS activated BMDMs from B6.TNF$^{-/-}$ mice than wild type controls (Fig 4A, 4B). The IFNγ treatment did not alter NO production of B6.WT, B6.TNF$^{-/-}$ macrophages after incubation with DFTD cells for 24 hours (Fig 4A, 4B). The release of NO might associate with the alteration of phagocytosis in IFNγ/LPS and LPS alone treated B6.TNF$^{-/-}$ macrophages.

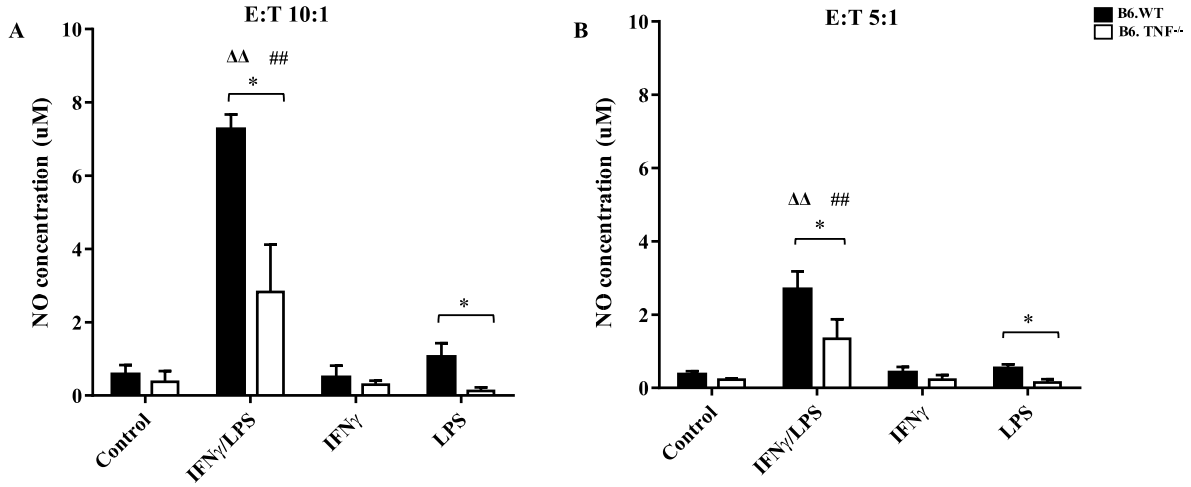

**Figure 4. Reduced NO production from IFNγ/LPS, LPS activated B6.TNF$^{-/-}$ macrophages phagocytosis of DFTD cells.** B6.WT and B6.TNF$^{-/-}$ macrophages were stimulated with IFNγ/LPS, IFNγ or LPS overnight. Macrophages were co-incubated with DFTD cells at 10:1(A) and 5:1 (B) ratios, the concentration of NO in the cell culture supernatant was analysed in Griess assay. Data were from 3 mice, error bars indicate SEM, ΔΔ P< 0.01 versus WT control, ## P< 0.01 versus B6.TNF$^{-/}$control, *P<0.05 versus WT versus B6.TNF$^{-/-}$.

**NO is not required for TNF regulation of macrophage phagocytosis of DFTD cells.**

To determine the association of NO with increased phagocytic activity in IFNγ/LPS and LPS alone treated B6.TNF$^{-/-}$ macrophages. The NO release was blocked by the inhibitor of nitric oxide synthesis, L-N6- (1-Iminoethyl) lysine dihydrochlorideinducible (L-NIL). After the incubation with IFNγ/LPS or IFNγ/LPS and L-NIL for 24 hours, BMDMs from B6.WT and B6.TNF$^{-/-}$ mice were co-incubated with DFTD cells for 24 hours. The number of DFTD cells was measured by flow cytometry and cell counting beads. As shown in Fig 5, L-NIL did not alter phagocytosis following stimulation with IFNγ/LPS of macrophages from B6.WT or B6. TNF$^{-/-}$ mice. In addition, compared to IFNγ/LPS treatment, L-NIL treatment did not alter the phagocytic ability of macrophages from B6.WT or B6.TNF$^{-/-}$ mice (Fig 5). Thus, it suggests that NO was unrelated to the altered phagocytic ability of IFNγ/LPS treated macrophages from B6.TNF$^{-/-}$ mice.

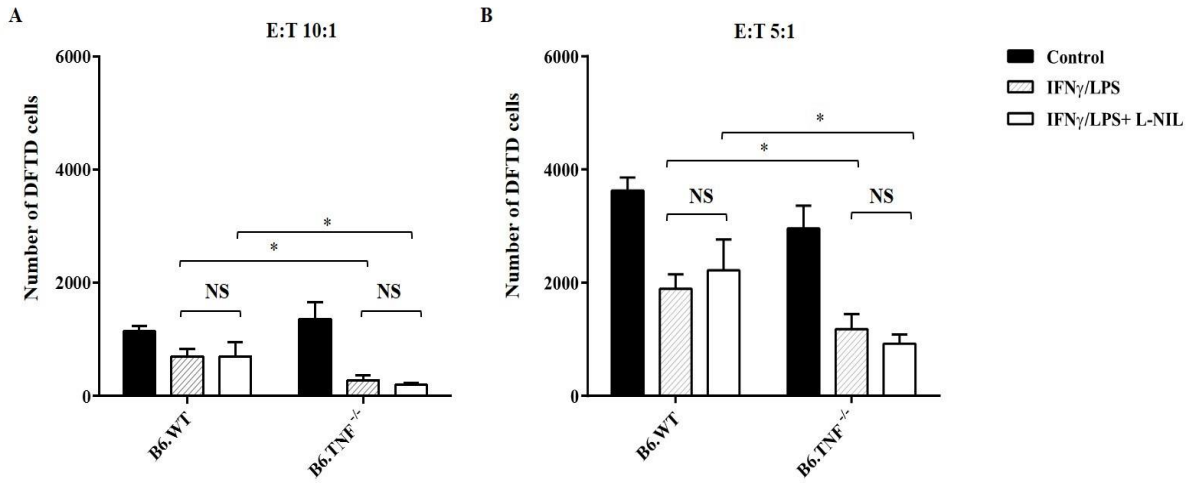


**Figure 5. NO is unrelated to TNF involved macrophage phagocytosis of DFTD cells.** B6.WT and B6.TNF$^{-/-}$ BMDMs were treated with IFNγ/LPS or IFNγ/LPS with L-NIL

overnight. After incubation with BMDMs and DFTD cells for 24 hours, the numbers of DFTD cells at E: T 10:1 (A), 5:1 (B) ratios were analysed by flow cytometry and cell counting beads. The numbers of DFTD cells were calculated as methods described. Data were from 3-5 mice of two or three independent experiments, error bars indicate SEM, * P< 0.05, NS not significantly.

**Discussion**

The key points of our study are that DFTD cells are phagocytosed effectively by bone marrow derived macrophages from mice and that TNF reduced this phagocytosis. The main consequences of these findings are two-fold. DFTD avoidance of phagocytosis is unlikely to contribute to immune escape. Secondly, TNF is not required for phagocytosis, but it appears to negatively regulate phagocytosis by activated macrophages.

The absence of MHC-I expression on DFT1 cells prevents the activation $CD8^+$ T cell adaptive immune response [12]. This would prevent an adaptive cell mediated immune response. An enhancement of innate immune response to DFTD such as macrophage phagocytosis may provide a mechanism to promote anti-DFTD immune responses. This could be either naturally or artificially such as vaccination. The evidence that immunised devils [20, 21] and some wild devils [22] can produce antibodies against DFTD cells is in line with antibody mediated opsonisation of DFT1 cells and subsequent phagocytosis. For this to occur, DFT1 cells would need the capacity to be phagocytosed. Tumour cells can avoid phagocytosis by expressing inhibitory molecules such as the ‘don't eat me signal’, which is part of the immune checkpoint inhibitory pathway, preventing phagocytosis [23]. RNA transcriptome analysis identified CD47 from devil mononuclear cells, which aligned closely with other species [24]. Hence there is the potential for DFT1 cells to express this molecule to avoid phagocytosis. In the absence of monoclonal antibodies to identify such molecules and the unavailability of devil macrophages, we conducted phagocytosis of DFT1 cells using bone marrow derived mouse macrophages. If mouse derived macrophages could phagocytose DFT1 cells, then devil macrophages should also have this ability. We showed the phagocytosis of DFTD cells by mouse macrophages by two independent mechanisms, flow cytometry and confocal microscopy. The confocal microscopy, provided the additional evidence of destruction of the DFT1 tumour cells. The susceptibility of DFT1 cells to phagocytosis has at least two important implications. Firstly, wild devils that have the capacity to make antibodies against DFT1 cells could promote phagocytosis of antibody opsonised DFT1 cells [22]. Secondly, devil

immunised with DFT1 cells that produce antibody [20, 21] could also opsonise the DFT1 cells and target them for phagocytosis. In both situations, promotion of a specific immune response could follow due to the interaction of the macrophages and cytokines produced during phagocytosis. This could result in cytokines upregulating MHC-I [12]and the DFT1 cells becoming targets for CD8 T cells, as shown with immunotherapy [20].

After determining that mouse macrophages effectively phagocytose DFT1 tumour cells we then investigated whether TNF was required. In the absence of stimulation, the presence of TNF made no difference to the phagocytic efficiency. But following activation with IFNγ/LPS or LPS (but not IFNγ), the presence of TNF reduced phagocytic activity. Although TNF is regarded as a master regulator of inflammatory cytokine production (reviewed in [25]) there are reports that TNF can inhibit macrophage phagocytic activity. Exogenous TNF has been shown to reduce the macrophage capacity to ingest apoptotic cells [26]. This inhibition appears specific for apoptotic cells as it is not seen with beads or antibody-opsonized cells [26]. The authors propose that within the inflammatory environment, the reduced phagocytic ability may 'contribute to the local intensity of the inflammatory response'. Macrophages stimulated with LPS produce TNF and this would inhibit macrophage phagocytosis of apoptotic cells in an autocrine manner [27]. TNF is also produced by IFNγ/LPS activated macrophages [28, 29]. IFNγ on its own is unable to activate macrophages to produce TNF, but can do so in the presence of LPS [30]. The situation can be somewhat murky as some studies showed IFNγ primed macrophages [6, 31] can produce TNF. Furthermore, IFNγ appears to restore an impaired ability of macrophages to phagocytose apoptotic cells in a nitric oxide-dependent manner that required TNF production [6, 31]. But these might be due to the macrophages coming from different sources and at different stages of maturation. For example, although TNF-α has no effect on phagocytosis by immature macrophages, it reduces phagocytosis by mature macrophages [26].

Phagocytosis of pathogens by macrophages requires NO and TNF dependent NO production is required for the enhanced phagocytosis of apoptotic cells by IFNγ activated macrophages [6]. However, NO can impair phagocytosis of fluoresecent particles by affecting cytoskeletal assembly and pseudopod formation [32]. TNF is definitely involved in NO production as it induces iNOS expression via NF- □B [19, 29]. Furthermore, the expression of iNOS in IFNγ/LPS activation macrophages requires TNF [33]. TNF dependent NO production is required for the enhanced phagocytosis of apoptotic cells by IFNγ activated macrophages [6]. We blocked NO release by using L-NIL, but inhibition of NO did not alter phagocytosis

efficiency in IFNγ/LPS treated macrophages. It suggests the NO is independent of in TNF related IFNγ/LPS treated macrophages. Moreover, actin polymerization is an essential event in the process of macrophage phagocytosis. It has been suggested that exogenous TNF decreases actin reorganization in J774 macrophages [34]. The involvement of actin and TNF inhibition of phagocytosis activity in IFNγ/LPS activated macrophages needs to be further investigated.

In this study, TNF is inhibitory in macrophage phagocytosis under activation. The orchestration of TNF in macrophage defense against pathogens maybe important in homeostasis.

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**References:**

1. Chu, W.M., *Tumor necrosis factor.* Cancer Lett, 2013. **328**(2): p. 222-5.
2. Aderem, A. and D.M. Underhill, *Mechanisms of phagocytosis in macrophages.* Annu Rev Immunol, 1999. **17**(1): p. 593-623.
3. Underhill, D.M. and A. Ozinsky, *Phagocytosis of microbes: complexity in action.* Annu Rev Immunol, 2002. **20**(1): p. 825-852.
4. Gordon, S. and F.O. Martinez, *Alternative activation of macrophages: mechanism and functions.* Immunity, 2010. **32**(5): p. 593-604.
5. Martinez, F.O., L. Helming, and S. Gordon, *Alternative activation of macrophages: an immunologic functional perspective.* Annu Rev Immunol, 2009. **27**: p. 451-483.
6. Fernandez-Boyanapalli, R., et al., *Impaired phagocytosis of apoptotic cells by macrophages in chronic granulomatous disease is reversed by IFN-γ in a nitric oxide-dependent manner.* J Immunol, 2010. **185**(7): p. 4030-4041.

7. Varin, A., et al., *Alternative activation of macrophages by IL-4 impairs phagocytosis of pathogens but potentiates microbial-induced signalling and cytokine secretion.* Blood, 2010. **115**(2): p. 353-362.
8. Borges, V.M., et al., *TNFα inhibits apoptotic cell clearance in the lung, exacerbating acute inflammation.* Am J Physiol Lung Cell Mol Physiol, 2009. **297**(4): p. L586-L595.
9. Leichtle, A., et al., *CC chemokine ligand 3 overcomes the bacteriocidal and phagocytic defect of macrophages and hastens recovery from experimental otitis media in TNF-/- mice.* J Immunol, 2010. **184**(6): p. 3087-97.
10. Hawkins, C., et al., *Emerging disease and population decline of an island endemic, the Tasmanian devil Sarcophilus harrisii.* Biol Conserv, 2006. **131**(2): p. 307-324.
11. Kreiss, A., et al., *A histological and immunohistochemical analysis of lymphoid tissues of the Tasmanian devil.* Anat Rec, 2009. **292**(5): p. 611-620.
12. Siddle, H.V., et al., *Reversible epigenetic down-regulation of MHC molecules by devil facial tumour disease illustrates immune escape by a contagious cancer.* PNAS, 2013. **110**(13): p. 5103-5108.
13. Körner, H., et al., *Distinct roles for lymphotoxin-and tumour necrosis factor in lymphoid tissue organogenesis and spatial organisation defined in gene targeted C57BL/6 mice.* Eur. J. Immunol, 1997. **27**: p. 2600-2609.
14. Pearse, A.-M., et al., *Evolution in a transmissible cancer: a study of the chromosomal changes in devil facial tumor (DFT) as it spreads through the wild Tasmanian devil population.* Cancer Genet, 2012. **205**(3): p. 101-112.
15. Wilhelm, P., et al., *Rapidly fatal leishmaniasis in resistant C57BL/6 mice lacking TNF.* J Immunol, 2001. **166**(6): p. 4012-9.
16. Zhou, Z. and X. Yu, *Phagosome maturation during the removal of apoptotic cells: receptors lead the way.* Trends Cell Biol, 2008. **18**(10): p. 474-85.
17. Tjelle, T.E., T. Løvdal, and T. Berg, *Phagosome dynamics and function.* Bioessays, 2000. **22**(3): p. 255-263.
18. MacMicking, J., Q.-w. Xie, and C. Nathan, *Nitric oxide and macrophage function.* Annu Rev Immunol, 1997. **15**(1): p. 323-350.
19. Ding, A.H., C.F. Nathan, and D.J. Stuehr, *Release of reactive nitrogen intermediates and reactive oxygen intermediates from mouse peritoneal macrophages. Comparison of activating cytokines and evidence for independent production.* J Immunol, 1988. **141**(7): p. 2407-2412.

20. Tovar, C., et al., *Regression of devil facial tumour disease following immunotherapy in immunised Tasmanian devils.* Sci Rep, 2017. **7**: p. 43827.
21. Kreiss, A., et al., *Evidence for induction of humoral and cytotoxic immune responses against devil facial tumor disease cells in Tasmanian devils (Sarcophilus harrisii) immunized with killed cell preparations.* Vaccine, 2015. **33**(26): p. 3016-3025.
22. Pye, R., et al., *Demonstration of immune responses against devil facial tumour disease in wild Tasmanian devils.* Biol Lett, 2016. **12**(10).
23. Alvey, C. and D.E. Discher, *Engineering macrophages to eat cancer: from "marker of self" CD47 and phagocytosis to differentiation.* J Leukoc Biol, 2017.
24. Flies, A.S., et al., *Comparative Analysis of Immune Checkpoint Molecules and Their Potential Role in the Transmissible Tasmanian Devil Facial Tumor Disease.* Front Immunol, 2017. **8**: p. 513.
25. Parameswaran, N. and S. Patial, *Tumor necrosis factor-alpha signaling in macrophages.* Crit Rev Eukaryot Gene Expr, 2010. **20**(2): p. 87-103.
26. McPhillips, K., et al., *TNF-alpha inhibits macrophage clearance of apoptotic cells via cytosolic phospholipase A2 and oxidant-dependent mechanisms.* J Immunol, 2007. **178**(12): p. 8117-26.
27. Feng, X., et al., *Lipopolysaccharide inhibits macrophage phagocytosis of apoptotic neutrophils by regulating the production of tumour necrosis factor α and growth arrest‐specific gene 6.* Immunology, 2011. **132**(2): p. 287-295.
28. Cohen, H.B., et al., *IFN-γ prevents adenosine receptor (A2bR) upregulation to sustain the macrophage activation response.* J Immunol, 2015. **195**(8): p. 3828-3837.
29. Patel, N.R., et al., *Cell elasticity determines macrophage function.* PloS one, 2012. **7**(9): p. e41024.
30. Gifford, G.E. and M.-L. Lohmann-Matthes, *Gamma interferon priming of mouse and human macrophages for induction of tumor necrosis factor production by bacterial lipopolysaccharide.* J Natl Cancer Inst 1987. **78**(1): p. 121-124.
31. Vila-del Sol, V., C. Punzón, and M. Fresno, *IFN-γ-induced TNF-α expression is regulated by interferon regulatory factors 1 and 8 in mouse macrophages.* J Immunol, 2008. **181**(7): p. 4461-4470.
32. Jun, C.D., et al., *Nitric oxide induces ADP-ribosylation of actin in murine macrophages:*

*association with the inhibition of pseudopodia formation, phagocytic activity, and adherence on a laminin substratum.* Cell Immunol, 1996. **174**(1): p. 25-34.

33. Chan, E.D. and D.W. Riches, *IFN-γ+ LPS induction of iNOS is modulated by ERK, JNK/SAPK, and p38 mapk in a mouse macrophage cell line.* Am J Physiol Lung Cell Mol Physiol, 2001. **280**(3): p. C441-C450.

34. Peppelenbosch, M., et al., *Multiple signal transduction pathways regulate TNF-induced actin reorganization in macrophages: inhibition of Cdc42-mediated filopodium formation by TNF*. J Immunol, 1999. **162**(2): p. 837-845.